Gary Stephens · Sumiko Mochida
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Preface

This book unites leading international experts to discuss recent advances in the regulation of mammalian presynaptic voltage-gated Ca^{2+} channels (VGCCs). It is now commonly realised that VGCC expression is a hallmark characteristic of an excitable cell. This book focuses on the involvement of VGCCs localised to neuronal presynapses and indispensible for chemical neurotransmission; the emphasis is on members of the Ca_V2 subunit family: $Ca_V2.1$ (P/Q-type Ca^{2+} current), $Ca_V2.2$ (N-type Ca^{2+} current) and $Ca_V2.3$ (R-type Ca^{2+} current); however, Ca_V1 (L-type Ca^{2+} current) subunits also have a role in neurotransmitter release at certain synapses, such as photoreceptors and auditory hair cells, in addition to functions in excitation-contraction coupling in muscle cells. Functions of the pore-forming $Ca_V\alpha$ subunits are supported by the expression of auxiliary VGCC subunits, predominantly $Ca_V\beta$ and $\alpha_2\delta$ subunits, which affect trafficking and gating of the VGCC complex and, as recent evidence suggests, also possess independent functionality. It is also becoming clear that VGCCs are involved in nuclear signalling, and, importantly, that VGCC subunits represent bona fide molecular targets for therapeutic drug discovery. The contributions to this book will cover these various facets as described below.

Calcium Channels in a Historical Context

It is over 60 years since the notion that Ca^{2+} could carry electrical signals was first suggested, somewhat tentatively, by Paul Fatt and Bernard Katz. It was a further 5 years before this work was followed up, when Fatt and Bernard Ginsborg published further studies in large crustacean muscle in 1958; the importance of Ca^{2+} as a second messenger was progressed mainly by Susumu Hagiwara's work using invertebrate giant muscle fibres. Although squid giant axons, at this time the most widely used preparation for studying electrical excitability, do not carry high levels of Ca^{2+} current, subsequent studies identified Na^+-independent Ca^{2+} action potentials and Ca^{2+} currents in several mammalian skeletal and

cardiac muscle preparations. The later introduction of the patch clamp technique was pivotal in advancing these earlier electrophysiological studies towards the recognition of membrane Ca^{2+} channel proteins; however, pharmacology also played an important role. The concept of 'calcium antagonism' was first posited by Albrecht Fleckenstein in the mid-1960s: blockers of Ca^{2+} influx into cardiac tissue (including dihydropyridine (DHP) agents that represent important therapeutic drugs today) were proposed to target 'calcium receptors'. Such DHPs were instrumental in providing bait for purifying and cloning the first $Ca_V\alpha$ subunits from the requisite rich protein source of skeletal muscle. Thus, biochemical work from several groups contributed to the description of the purified DHP receptor complex, comprising five molecular components: α (170 kDa), α2 (150 kDa), β (52 kDa), δ (17–25 kDa) and γ (32 kDa) subunits, with the pore-forming, DHP-binding α subunit being cloned by the Numa group in 1987.

Alongside this elegant biochemical and molecular biological work, it soon became apparent from whole-cell and single-channel electrophysiological recordings that multiple Ca^{2+} currents exist; initially these were subdivided into two main types, which later came to be known as low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca^{2+} currents. Work from Richard Tsien's laboratory in the mid-1980s further refined the HVA division, distinguishing large conductance L-type Ca^{2+} current from neuronal N-type current; again justification came from biophysical and pharmacological evidence. In this regard, isolation of ω-conotoxin, a peptide toxin from the *Conus* sea-snail, helped to fully identify the role of N-type current in transmitter release at peripheral and central synapses. Work from the Llinás laboratory added the P-type Ca^{2+} current in 1989, originally recorded in isolated Purkinje neurons and found to be blocked by the funnel web spider toxin ω-agatoxin IVA, and the variant Q-type current was described by the Tsien laboratory. Finally, an R-type Ca^{2+} current, resistant to L-, N and P/Q pharmacological blockers was described by Randell and Tsien in 1995. This R-type Ca^{2+} current was subsequently shown to be sensitive to SNX-482, a synthetic peptide originally isolated from tarantula spider venom.

In the early 1990s, molecular cloning techniques allowed a fuller definition of the channel proteins underlying neuronal Ca^{2+} currents. The brain B1 channel (later termed α1A and shown to be responsible for P/Q-type current) was cloned by the Numa group in 1991. This was closely followed by the cloning of the α1B subunit, responsible for N-type current in 1992, and the α1E subunit, responsible for R-type current in 1993, by the Snutch group. The elusive molecular counterparts of the LVA (T-type) channels were finally cloned in silico by the Perez-Reyes group. Thus, ten genes underlying Ca^{2+} currents have been subdivided into a revised nomenclature: $Ca_V1.1$-$Ca_V1.4$ (α1S, α1C, α1D and α1F, L-type); $Ca_V2.1$ (α1A, P/Q); $Ca_V2.2$ (α1B, N-type); $Ca_V2.3$ (α1E, R-type); $Ca_V3.1$–$Ca_V3.3$ (α1G, α1H and α1I, T-type). The auxiliary $Ca_V\beta$ ($Ca_V\beta_{1–4}$) and the $Ca_V\alpha_2\delta$ ($Ca_V\alpha_2\delta_{1–4}$) subunits have subsequently been cloned. Throughout this book, the IUPHAR recognised nomenclature and description of 'voltage-gated calcium (Ca^{2+}) channels (VGCCs)' is used (although description of voltage-dependent and voltage-activated

calcium (Ca^{2+}) channels are also common in the literature), and the revised terminology for the pore-forming subunit is typically used in preference to the older '$\alpha 1$ subunit' nomenclature. The term '$Ca_V\beta$ subunits' is used to specify those subunits associated with the VGCC complex or, sometimes, more simply 'β subunits' when this description is unambiguous. Finally, here, the term $\alpha_2\delta$ subunits or, occasionally, $Ca_V\alpha_2\delta$ subunits, is used largely dependent on author preference.

Contents of This Volume

At the presynapse, VGCCs are crucial for the translation of action potential-driven changes in membrane potential to the fast, synchronous release of neurotransmitter via the generation of localised increases in intraterminal Ca^{2+} concentration. The unique requirement for VGCC activation in the conversion of electrical signal to a chemical message is almost universal for every type of excitable cell. It has also become clear more recently that VGCCs may also contribute to spontaneous transmitter release at certain presynaptic terminals. In addition to fast transmitter release, VGCCs are also vital for presynaptic plasticity. The spatial and temporal modulation of VGCCs by a range of synaptic, Ca^{2+} binding and regulatory proteins is discussed in the introductory Chap. 1 by Sumiko Mochida.

Modulation of Calcium Channels by Binding Partners

VGCCs are one of the most widely modulated groups of protein in the body, being the target of a range of effector pathways and representing an extended signalling complex, as exemplified by the recent description of the extensive $Ca_V2.2$ proteome. An important redundancy in VGCC regulation by these binding partners exists, which likely provides security for physiological function. Presynaptic VGCC function is highly dependent on the co-expression of auxiliary subunits, predominantly the intracellular $Ca_V\beta$ subunit and the transmembrane $\alpha_2\delta$ subunit; both species have been shown to affect trafficking to the presynaptic membrane and gating of the VGCC complex and, also, to have potential roles in neuronal disease, as discussed by Gerald Obermair and Bernd Flucher in Chap. 2.

VGCC are localised to presynaptic active release zones via PDZ-containing binding partners, where they supply Ca^{2+} to sensors for exocytosis; the coupling between VGCCs and Ca^{2+} sensors may be via microdomains (>100 nm) or, as recent evidence has shown for several synapses, via nanodomains (<100 nm). Within such domains, Ca^{2+} dependent protein-protein interactions ensure delivery and fusion of synaptic vesicles and correct release of their transmitter contents. The reciprocal regulation of VGCCs by proteins of the synaptic core complex is discussed by Norbert Weiss and Gerald Zamponi in Chap. 3. The following

contribution combines previous themes; thus, in Chap. 4, Akito Nakao, Mitsuru Hirano, Yoshinori Takada, Shigeki Kiyonaka and Yasuo Mori discuss the fine-tuning of neurotransmitter release by active zone proteins and, in particular, how such proteins are themselves regulated by auxiliary $Ca_V\beta$ subunits.

Another major pathway by which neurotransmission is modulated is by G protein regulation of presynaptic VGCC function; this area is reviewed by Mark Jewell and Kevin Currie (Chap. 5). The modulation of Ca_V2 VGCCs by $G\beta\gamma$ subunits and the influence of other interacting proteins, including $Ca_V\beta$ subunits and SNARE proteins, are considered. The role of the Ras-related GTP-binding protein family RGK as small GTPases that regulate Ca_V1, and also $Ca_V2.1$ and $Ca_V2.2$ and, interestingly, $Ca_V\beta$ function at the presynapse, is discussed by Pierre Charnet, Frédérique Scamps, Matthieu Rousset, Claudine Menard, Michel Bellis and Thierry Cens in Chap. 6.

Whilst $Ca_V2.1$ and $Ca_V2.2$ are routinely described as the major VGCCs associated with the presynaptic active zone, the $Ca_V2.3$ subunit, which underlies R-type Ca^{2+} current, can also contribute to neurotransmitter release, perhaps at more adjacent sites. In Chap. 7, Maxine Dibué, Etienne Tevoufouet, Andreas Krieger, Alexandra Kiel, Dimitar Evdokimov, Thomas Galetin, Serdar Alpdogan, Isha Akhtar, Sabrina Scharf, Renate Clemens, Kayalvizhi Radhakrishnan, Jürgen Hescheler, Toni Schneider and Marcel Kamp describe potential functions of this 'enigmatic' VGCC in terms of novel protein interaction partners.

Finally in this section (Chap. 8), the cell nucleus is included as a signalling partner for the VGCC. Michel Bellis, Thierry Cens, Claudine Menard, Pierre Charnet and Matthieu Rousset focus on the process of excitation-transcription coupling, whereby opening of VGCCs leads to specific activity-regulated transcription programmes in the nucleus of presynaptic or postsynaptic cells, as a novel form of signalling that has parallels with neurosecretion.

Mechanisms of Studying Calcium Channel Effects

In this section, the use of specialised techniques and how they have added to our overall knowledge of VGCC function is considered. A major advance has been in the development of direct electrophysiology recordings from mammalian presynaptic terminals. In Chap. 9, Holger Taschenberger, Kun-Han Lin and Shuwen Chang discuss their recent work using the calyx of Held, a large glutamatergic CNS terminal that has been instrumental in increasing our knowledge of presynaptic VGCC function; in particular, the authors focus on processes of short-term plasticity. Next, we discuss the use of synthetic Ca^{2+} channel peptides to study presynaptic function (Chap. 10). We review work with the 'synprint' peptide based on the intracellular loop connecting $Ca_V2.2$ domains II and III, and discuss our recent work with $Ca_V2.2$ amino terminal and I–II loop peptides, including the effects of direct presynaptic introduction of such peptides.

Calcium Channel Therapeutics

In the final section, the potential therapeutic targeting of VGCCs are considered, both in terms of the pore-forming $Ca_V\alpha$ subunit and the rise to prominence of the $\alpha_2\delta$ subunit, the binding partner of the major anti-convulsant gabapentinoids, agents that are now also first-line treatments for neuropathic pain. In the mature CNS, neurotransmitter release is predominantly mediated by the $Ca_V2.1$ subunit. Several spontaneous mutant mouse models present disease phenotypes which can be used to model human neurological conditions. In Chap. 11, David Friel considers the *leaner* mouse model, examining the effects of the loss-of-function $Ca_V2.1$ mutation on excitatory synaptic transmission in the cerebellum. In a similar fashion, Osvaldo Uchitel reviews how the study of $Ca_V2.1$ dysfunction due to channel mutations (so-called channelopathies) may aid future therapeutic development (Chap. 12); effects of mutations on $Ca_V2.1$ function in rare hereditary forms of migraine and ataxia and, also, in autoimmune disorders such as myasthenic syndrome and amyotrophic sclerosis are further considered. Hua Huang, Juejin Wang and Tuck Wah Soong discuss genetic knock-out, mutations and, in particular, alternative splicing and RNA editing of VGCCs and their implications for therapeutics (Chap. 13). Edward Stevens and Peter Cox review the targeting of VGCCs for treatment of pain from a pharmaceutical industry viewpoint in Chap. 14. The development and clinical introduction of conotoxin-based $Ca_V2.2$ blockers such as ziconotide are considered, together with the development of gabapentinoids to target $\alpha_2\delta_1$ subunit. Finally, Chunyi Zhou and Z. David Luo extend the themes of the previous chapter to discuss how models of chronic, neuropathic pain have implicated the $\alpha_2\delta_1$ subunit as a major drug target (Chap. 15).

Together, these expert contributions provide a thorough review of recent work in the high profile and exciting field of VGCC research and, more importantly, provide compelling evidence that, as with any truly fundamental process, we still have much to learn about the role of these crucial proteins, their interactions and signalling pathways at presynaptic terminals. The work described here provides the basis for such research.

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Contents

Part I
Overview

Chapter 1
Overview: Spatial and Temporal Regulation of Ca^{2+} Channels

Sumiko Mochida

Abstract Neuronal firing activity induces membrane depolarization and subsequent Ca^{2+} entry through voltage-gated Ca^{2+} (Ca_V) channels that triggers neurotransmitter release at the presynaptic terminal. Presynaptic Ca^{2+} channels form a large signaling complex, which targets synaptic vesicles to Ca^{2+} channels for efficient release and mediates Ca^{2+} channel regulation. The presynaptic Ca_V2 channel family (comprising $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ isoforms) encode the pore-forming α1 subunit. The cytoplasmic regions are the target of regulatory proteins for channel modulation. Modulation of presynaptic Ca^{2+} channels has a powerful influence on synaptic transmission. This chapter overviews spatial and temporal regulation of Ca^{2+} channels by effectors and sensors of Ca^{2+} signaling, and describes the emerging evidence for a critical role of Ca^{2+} channel regulation in control of synaptic transmission and presynaptic plasticity. Sympathetic superior cervical ganglion neurons in culture expressing $Ca_V2.2$ channels represent a well-characterized system for investigating synaptic transmission. The exogenously expressed α1 subunit of the $Ca_V2.1$ as well as endogenous $Ca_V2.2$ was examined for modulation of channel activity, and thereby regulation of synaptic transmission. The constitutive and Ca^{2+}-dependent modulation of $Ca_V2.1$ channels coordinately act as spatial and temporal molecular switches to control synaptic efficacy.

Keywords Ca^{2+} channels • Synaptic transmission • G-proteins • Synaptic proteins • Ca^{2+} binding proteins

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1.1 Introduction

Neuronal firing activity induces membrane depolarization and subsequent Ca^{2+} entry through voltage-gated Ca^{2+} (Ca_V) channels that triggers neurotransmitter release at the presynaptic terminal. Multiple mechanisms directly or indirectly modulate the function of presynaptic Ca^{2+} channels (Snutch and Reiner 1992; Dunlap et al. 1995; Tedford and Zamponi 2006; Catterall and Few 2008). The ability of Ca_V channels to open, close, or inactivate in response to membrane depolarization changes temporally during and after neuronal firing activity and alters efficacy of synaptic transmission (Tedford and Zamponi 2006; Catterall and Few 2008). Following brief overviews of Ca^{2+} channel structure/function, this chapter reviews progress toward understanding the cellular and molecular mechanisms that modulate the activity of presynaptic Ca^{2+} channels, regulate synaptic transmission, and induce short term synaptic plasticity. I focus here on the spatial and temporal regulation of Ca^{2+} channels that have been shown to regulate synaptic transmission in functional synapses, including regulation by G protein coupled receptors, SNARE proteins, and residual intracellular Ca^{2+}.

Evidence in a large presynaptic terminal of the calyx of Held, where multiple Ca_V2 channels are expressed, provides a starting point to understand roles in modulation of presynaptic Ca^{2+} channels to regulate synaptic transmission in the central neurons. Sympathetic superior cervical ganglion (SCG) neurons form a well-characterized cholinergic synapse in long-term culture (Mochida et al. 1994; Ma and Mochida 2007), and synaptic transmission is mediated by $Ca_V2.2$ channels (Mochida et al. 1995, 2003a, b). The SCG neurons are an ideal cell model for $Ca_V2.1$ and 2.2 modulation (Mochida et al. 1996, 2003a, b, 2008; Stephens and Mochida 2005; Bucci et al. 2011). The large cell body and nucleus allow for the manipulation of gene expression and function in mature neurons via acute microinjection of cDNA, small interfering RNA (siRNA), dominant-negative transgenes, peptides, antibodies, and metabolites (Mochida et al. 2003a, b, 2008; Baba et al. 2005; Krapivinsky et al. 2006; Ma and Mochida 2007), an approach not technically feasible for cultured neurons from the central nervous system. In addition, synaptic activity and short-term plasticity can be accurately monitored by recording excitatory postsynaptic potentials (EPSPs) evoked by paired or repetitive action potentials in presynaptic neurons. Using this approach, we have uncovered a critical role of cytoplasmic N-terminal and I-II loop interaction (Bucci et al. 2011) and calmodulin (CaM), CaM-like Ca^{2+}-sensor proteins and CaMKII binding to the cytoplasmic C-terminal (Mochida et al. 2008; Leal et al. 2012; Magupalli et al. 2013) in regulation of Ca^{2+} channel activity, thus highlighting molecular mechanisms through which presynaptic function and plasticity are regulated by Ca^{2+} channel modulation. Cytoplasmic N-terminal and I-II loop interaction spatially modulate Ca^{2+} channel activity (Bucci et al. 2011). CaM is a Ca^{2+} effector sensing residual Ca^{2+} that mediates time- and space-dependent synaptic depression and facilitation via effects on Ca_V2 channel gating (Mochida et al. 2008; Leal et al. 2012). $Ca_V2.1$ channel has an 'effector checkpoint' associating with CaMKII to control channel fitness for function (Magupalli et al. 2013).

1.2 Presynaptic Ca^{2+} Channels

Ca^{2+} currents in different cell types have diverse physiological roles and pharmacological properties, and an alphabetical nomenclature has evolved for the distinct classes of Ca^{2+} currents (Tsien et al. 1988). N-type, P/Q-type, and R-type Ca^{2+} currents require strong depolarization for activation (Tsien et al. 1991) and are blocked by specific polypeptide toxins from snail and spider venoms (Miljanich and Ramachandran 1995). N-type and P/Q-type Ca^{2+} currents are observed primarily in neurons, where they initiate neurotransmission at most fast conventional synapses (Olivera et al. 1994; Dunlap et al. 1995; Catterall 2000). The Ca^{2+} channels that have been characterized biochemically are composed of four or five distinct subunits (Fig. 1.1) (Takahashi et al. 1987; Catterall 2000). The α1 subunit of 190–250 kDa is the largest subunit, and it incorporates the conduction pore, the voltage sensors and gating apparatus, and most of the known sites of channel regulation by second messengers, drugs, and toxins. The α1 subunit is composed of about 2,000 amino acid residues organized in four homologous domains (I–IV). Each domain of the α1 subunit consists of six transmembrane α helices (S1 through S6) and a membrane-associated P loop between S5 and S6. The S1 through S4 segments serve as the voltage sensor module (Fig. 1.1), whereas transmembrane segments S5 and S6 in each domain and the P loop between them form the pore module (Fig. 1.1) (Yu et al. 2005). The large intracellular segments of Ca^{2+} channels serve as a signaling platform for Ca^{2+}-dependent regulation of neurotransmission, as discussed below.

Ca^{2+} channel α1 subunits are encoded by ten distinct genes in mammals, which are divided into three subfamilies by sequence similarity (Snutch and Reiner 1992; Catterall 2000; Ertel et al. 2000). Division of Ca^{2+} channels into these three subfamilies is phylogenetically ancient, as single representatives of each are found in the *C. elegans* genome. The Ca_V2 subfamily members ($Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$) conduct P/Q-type, N-type, and R-type Ca^{2+} currents, respectively (Snutch and Reiner 1992; Olivera et al. 1994; Catterall 2000; Ertel et al. 2000).

The α1 subunits are associated with four distinct auxiliary protein subunits (Catterall 2000) (Fig. 1.1). The intracellular β subunit is a hydrophilic protein of 50–65 kDa. The transmembrane, disulfide-linked α2δ subunit complex is encoded by a single gene, but the resulting prepolypeptide is posttranslationally cleaved and disulfide-bonded to yield the mature α2 and δ subunits. A γ subunit having four transmembrane segments is a component of skeletal muscle Ca^{2+} channels, and related subunits are expressed in heart and brain. The auxiliary subunits of Ca^{2+} channels have an important influence on their function (Hofmann et al. 1999; Dolphin 2003). Ca_Vβ subunits greatly enhance cell surface expression of the α1 subunits and shift their kinetics and voltage dependence of activation and inactivation. The α2δ subunits also enhance cell surface expression of α1 subunits (Davies et al. 2007), and set presynaptic release probability (Hoppa et al. 2012). The γ subunits modulate cardiac Ca^{2+} channel function together with the β subunit (Yang et al. 2011). The functional role of the γ subunits of Ca^{2+} channels is the least well-defined. Although these four auxiliary subunits modulate the functional

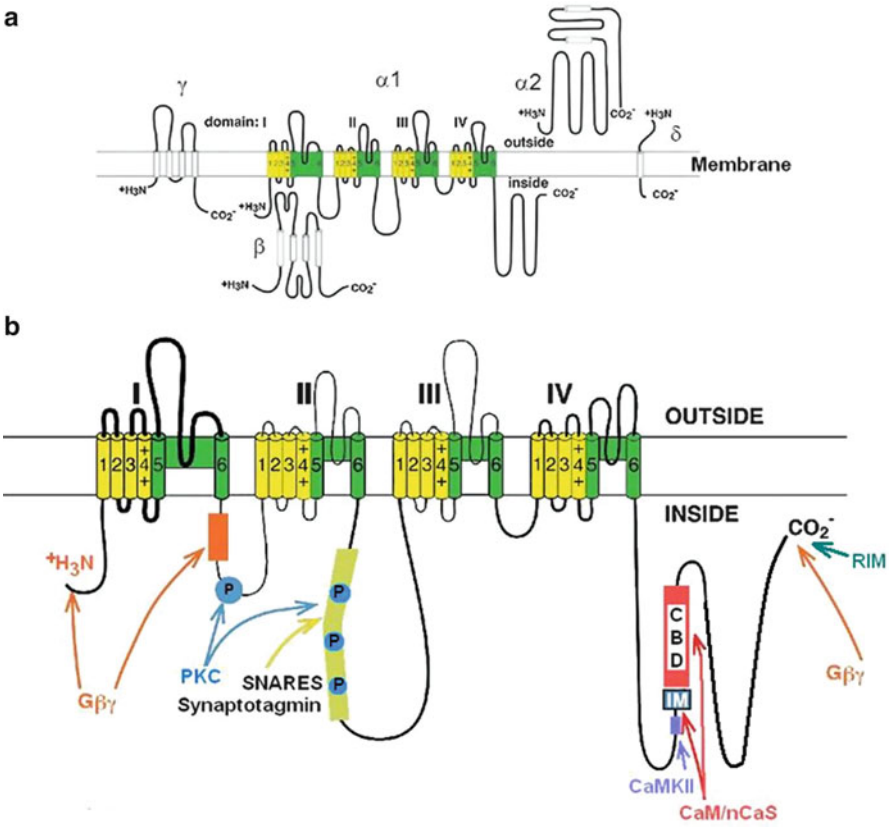

Fig. 1.1 Subunit Structure of Ca_V Channels. (**a**) The subunit composition and structure of high-voltage-activated Ca^{2+} channels. Predicted helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented. The voltage-sensing module is illustrated in yellow and the pore-forming module in green. (**b**) The sites of interaction of different regulatory proteins on the intracellular surface of the α_1 subunit of Ca_V2 channels (Adapted from Catterall and Few 2008)

properties of the Ca^{2+} channel complex, the pharmacological and physiological diversity of Ca^{2+} channels arises primarily from the existence of multiple α1 subunits.

1.3 Modulation of Presynaptic Ca^{2+} Channel Activity

1.3.1 Interaction with G Proteins

Most neurotransmitters, including acetylcholine, glutamate, GABA, biogenic amines, and many neuropeptides inhibit presynaptic N-type and P/Q-type Ca^{2+}

currents through activation of G protein-coupled receptors in nerve terminals (Hille 1994; Ikeda and Dunlap 1999). The most prominent form of G protein-induced inhibition causes a positive shift in the voltage dependence of activation of the Ca^{2+} current (Marchetti et al. 1986; Tsunoo et al. 1986; Bean 1989). Gβγ subunits released from heterotrimeric G proteins of the Gi/Go class are responsible for this form of Ca^{2+} channel inhibition (Hille 1994; Ikeda and Dunlap 1999). Gβγ binds directly to the N-type Ca^{2+} channel α1 subunits (Herlitze et al. 1996; Ikeda 1996) at three sites; the N terminus $_{45–55}$ (Canti et al. 1999), the intracellular loop connecting domains I and II (LI-II) $_{377–393}$ (Herlitze et al. 1997; Zamponi et al. 1997), and the C terminus$_{2257–2336}$ (Li et al. 2004). The sites in the N terminus and loop I-II exert the most potent effects. The Gβγ-induced inhibition can be reversed by strong positive depolarization (Marchetti et al. 1986; Tsunoo et al. 1986; Bean 1989). Reversal of this inhibition by depolarization provides a point of intersection between chemical and electrical signal transduction at the synapse and can potentially provide novel forms of short-term synaptic plasticity that do not rely on residual Ca^{2+} (see Sect. 1.6).

In addition to this voltage-dependent inhibition of Ca_V2 channels by direct interaction with G proteins, many neurons also exhibit voltage-independent inhibition of Ca_V2 channels that is dependent on intracellular signaling pathways and involves multiple protein kinases (Hille 1994; Dunlap et al. 1995; Strock and Diverse-Pierluissi 2004). Voltage-independent regulation by G proteins often involves the Gq family of G proteins, which regulate the levels of phosphatidylinositide lipids by inducing hydrolysis of phosphatidylinositol bisphosphate via activation of phospholipase C enzymes (Delmas et al. 2005). Through this pathway, transmitter release from rat sympathetic neurons via presynaptic muscarinic acetylcholine receptors is inhibited (Kubista et al. 2009).

Regulation of the $Ca_V2.2$ channels also involves interplay between Ca^{2+} channels and G protein interaction. Syntaxin-1A, a presynaptic plasma membrane protein, is required for G protein inhibition of presynaptic Ca^{2+} channels (Stanley and Mirotznik 1997), as cleavage of syntaxin-1A by botulinum toxin prevents G protein modulation of presynaptic Ca^{2+} channels in chick calyx synapses. Physical interactions between syntaxin-1A and Ca^{2+} channels is a prerequisite for tonic Gβγ modulation of $Ca_V2.2$ channels (Jarvis et al. 2000).

1.3.2 Interaction of Cytoplasmic Sites

The $Ca_V2.2$ α1 subunit contains several inhibitory interaction sites for $G\beta\gamma$ subunits, including the amino terminal (NT) and I–II loop. The NT and I–II loop have also been proposed to undergo a G protein-gated inhibitory interaction, while the NT itself has also been proposed to suppress Ca_V2 channel activity. Bucci et al. (2011) investigated the effects of $Ca_V2.2_{45–55}$ 'NT peptide' and a I–II loop α interaction domain ($Ca_V2.2_{377–393}$) 'AID peptide' on Ca^{2+} channel activity and G protein modulation in SCG neurons. Injection of NT or AID peptide into

SCG neuron synapses attenuated noradrenaline-induced G protein modulation and inhibited synaptic transmission. In isolated SCG neurons, NT and AID peptides reduced whole-cell Ca^{2+} current amplitude, modified voltage dependence of Ca^{2+} channel activation and attenuated noradrenaline-induced G protein modulation. Co-application of NT and AID peptide negated inhibitory actions. These finding suggest direct peptide interaction with presynaptic Ca^{2+} channels, with effects on current amplitude and gating representing likely mechanisms responsible for inhibition of synaptic transmission. Mutation within NT abolished inhibitory effects of the NT peptide (Bucci et al. 2011), suggesting that the $Ca_V2.2$ N-terminal and I–II loop contribute molecular determinants for Ca^{2+} channel function; the data favor a direct interaction of peptides with Ca^{2+} channels to inhibit synaptic transmission and attenuate G protein modulation.

1.3.3 Interaction with Active Zone Proteins

Rab-interacting molecule (RIM), an active zone protein that is required for vesicle docking and priming (Koushika et al. 2001; Schoch et al. 2002; Gracheva et al. 2008; Deng et al. 2011; Han et al. 2011; Kaeser et al. 2011), and is implicated in synaptic plasticity (Castillo et al. 2002; Schoch et al. 2002), interacts with the C-terminal cytoplasmic tails of $Ca_V2.1$ and 2.2 channels (Coppola et al. 2001; Hibino et al. 2002; Kaeser et al. 2011) (Fig. 1.1). The interaction of RIM with Ca^{2+} channel is essential for recruiting Ca^{2+} channels to presynaptic active zone (Kaeser et al. 2011) and determines channel density and vesicle docking at presynaptic active zone (Han et al. 2011). RIM-binding protein, RIM-BPs also interacts with $Ca_V2.1$ and 2.2 channels (Hibino et al. 2002). The tripartite complex composed of RIM, RIM-BPs and C-terminal tails of the Ca_V2 channels regulate the recruitment of Ca_V2 channels to active zones. RIM also interacts with $Ca_V\beta$ subunits and shifts the voltage dependence of inactivation to more positive membrane potentials, increasing Ca^{2+} channel activity (Kiyonaka et al. 2007). Regulation of presynaptic Ca^{2+} channel function and vesicle docking by RIM provides an additional potential pathway to increase the release probability of synaptic vesicles docked close to Ca_V2 channels.

1.3.4 Interaction with t-SNAREs

Synaptic vesicle (v)-SNARE synaptobrevin 2 and presynaptic plasma membrane (t)-SNAREs syntaxin-1 and SNAP-25 are required for fusion of synaptic vesicle with a plasma membrane to release neurotransmitters (Sudhof 2004). Both $Ca_V2.1$ and $Ca_V2.2$ channels colocalize densely with syntaxin-1 at the presynaptic nerve terminals (Cohen et al. 1991; Westenbroek et al. 1992, 1995). These channels can be isolated as a complex with SNARE proteins from central neurons (Bennett et al. 1992; Yoshida et al. 1992; Leveque et al. 1994). The t-SNARE proteins syntaxin-1A and SNAP-25, but not the v-SNARE synaptobrevin, specifically interact with

the $Ca_V2.2$ channel by binding to the intracellular loop between domains II and III (LII-III) of the $\alpha_1 2.2$ subunit$_{718–963,}$ named as the synprint site (Fig. 1.1) (Sheng et al. 1994). This interaction is Ca^{2+} dependent, with maximal binding at 20 μM Ca^{2+} and reduced binding at lower or higher Ca^{2+} concentrations (Sheng et al. 1996), suggesting sequential steps of association and dissociation of SNARE proteins with Ca_V2 channels as a function of Ca^{2+} concentration. Two peptide segments separated by a flexible linker within the synprint site independently bind both syntaxin-1A and SNAP-25 (Yokoyama et al. 2005). $Ca_V2.1$ channels have an analogous synprint site, and different channel isoforms have distinct interactions with syntaxin and SNAP-25 (Rettig et al. 1996; Kim and Catterall 1997), which may confer specialized regulatory properties that contribute to synaptic modulation.

Through interaction with $Ca_V2.1$ and $Ca_V2.2$ channels, presynaptic t-SNAREs regulate Ca^{2+} channel function. Coexpression of syntaxin-1A and/or SNAP-25 with $Ca_V2.1$ or $Ca_V2.2$ channels shifts the voltage dependence of inactivation toward more negative membrane potentials and reduces the availability of the channels to open and (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999). Coexpression of SNAP-25 can reverse the inhibitory effects of syntaxin on $Ca_V2.2$ channels (Wiser et al. 1996; Jarvis and Zamponi 2001). The synprint site binds to the entire H3 helix in the cytoplasmic domain of syntaxin-1A (Sheng et al. 1994, 1996; Bezprozvanny et al. 2000). However, the transmembrane region and only a short segment within the H3 helix are critical for channel modulation (Bezprozvanny et al. 2000). Deletion of the synprint site weakened the modulation of the channels by syntaxin-1A, but did not abolish it, arguing that the synprint site acts as an anchor in facilitating channel modulation but is not required absolutely for modulatory action.

1.3.5 Interaction with Synaptotagmin-1

Synaptotagmin-1, -2, -3, and -9 serve as the Ca^{2+} sensors for the fast, synchronous neurotransmitter release (Geppert et al. 1994; Sudhof 2004; Xu et al. 2007). Synaptotagmin-1 contains two homologous C2 domains, which bind Ca^{2+} to initiate synchronous transmitter release (Sudhof 2004). The C2B domain of synaptotagmin-1 binds to the synprint sites of both $Ca_V2.1$ and $Ca_V2.2$ channels (Sheng et al. 1997). Moreover, syntaxin-1 interacts competitively with either synprint or synaptotagmin-1 in a Ca^{2+}-dependent manner, such that at low Ca^{2+} concentrations syntaxin-1 binds synprint, whereas at higher concentrations (>30 μM) its association with synaptotagmin-1 increases. The sequential Ca^{2+}-dependent binding of syntaxin-1 to the synprint site and then to synaptotagmin-1 *in vitro* may reflect stepwise protein interactions that occur during exocytosis (Sheng et al. 1996). Coexpression of synaptotagmin-1 can relieve the inhibitory effects of SNAP-25 on $Ca_V2.1$ channels (Wiser et al. 1997; Zhong et al. 1999). Relief of Ca^{2+} channel inhibition by formation of a complete synaptotagmin/SNARE complex favors Ca^{2+} influx through Ca_V2 channels, thus providing a potential mechanism to increase the release probability of synaptic vesicles that are docked close to Ca_V2 channels (Catterall and Few 2008).

1.3.6 Regulation of Synprint Site by Protein Kinases

Several protein kinases are localized in presynaptic terminals and phosphorylate Ca^{2+} channels and SNARE proteins. Phosphorylation of the synprint peptide by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) *in vitro* strongly inhibits its binding to syntaxin-1A and SNAP-25 (Yokoyama et al. 1997). The two separate segments of the synprint site that each bind syntaxin-1 and SNAP-25 *in vitro* are regulated by PKC phosphorylation at serines 774 and 898 and by CaMKII phosphorylation at serines 784 and 896, respectively (Yokoyama et al. 2005). Each phosphorylation site controls syntaxin-1 and SNAP-25 binding to half of the synprint site (Yokoyama et al. 2005). PKC phosphorylation blocks the negative shift of steady-state inactivation of $Ca_V2.2$ channels caused by syntaxin (Jarvis and Zamponi 2001; Yokoyama et al. 2005). These studies suggest that phosphorylation of the synprint site by PKC or CaMKII may serve as a biochemical switch controlling the SNARE-synprint interaction. This mechanism provides a potential functional link between neurotransmitter-activated protein phosphorylation and tethering docked synaptic vesicles in an optimal position to respond to the Ca^{2+} signal from presynaptic Ca^{2+} channels (Catterall and Few 2008).

1.3.7 Regulation of Ca^{2+} Channel by Ca^{2+} and Calmodulin

During trains of depolarizations, P/Q-type Ca^{2+} currents increase in size during the first pulses due to facilitation and then inactivate in a pulsewise manner (Cuttle et al. 1998; Lee et al. 2000). Both facilitation and inactivation are prevented when Ba^{2+} is the permeant ion and when Ca^{2+} is rapidly chelated by BAPTA. However, inactivation, but not facilitation, is prevented by a high intracellular concentration of EGTA (10 mM) (Lee et al. 2000). These results indicate that the facilitation process has higher affinity and/or more rapid binding of Ca^{2+} than the inactivation process. Both Ca^{2+}-dependent facilitation and inactivation of $Ca_V2.1$ channels are dependent on calmodulin (CaM) (Lee et al. 1999, 2000; DeMaria et al. 2001). In the C-terminal domain of the $Ca_V2.1$ subunit, CaM interacts with a modified IQ-like motif, which begins with the sequence isoleucine$_{1913}$-methionine$_{1914}$ (IM) rather than isoleucine-glutamine (IQ), and with a second nearby downstream site, the CaM binding domain, ($CBD_{1969–2000}$) (Lee et al. 1999, 2003; DeMaria et al. 2001). Ca^{2+}-dependent facilitation is impaired by mutations in CaM that prevent binding of Ca^{2+} at the C-terminal EF-hands (DeMaria et al. 2001; Lee et al. 2003). In contrast, Ca^{2+}-dependent inactivation is preferentially inhibited by mutations of the Ca^{2+}-binding sites in the N-terminal lobe of CaM (DeMaria et al. 2001; Lee et al. 2003). Studies using multiphoton microscopy and a microfluidic mixer have revealed two sequential, rapid conformation changes of CaM upon binding Ca^{2+}, which may be the molecular basis for its biphasic regulation of Ca^{2+} channel function

(Park et al. 2008). The first transition in the C-terminal lobe proceeds with a time constant of 0.5 ms. The second transition in the N-terminal lobe proceeds with a time constant of 20 ms. These absolute rate constants are faster than facilitation and inactivation of $Ca_V2.1$ channels, but these lobe-specific conformational transitions in CaM would be expected to be slowed by its binding to a regulatory target in which it must induce additional conformational changes as part of its regulatory mechanism. The 40-fold difference in the rates of the two conformational changes in CaM approximates the difference in rates of facilitation and inactivation, supporting the idea that they may indeed represent the molecular mechanism for biphasic regulation of $Ca_V2.1$ channels.

The two lobes of CaM interact differentially with the two CaM binding subsites in the C-terminal domain of $Ca_V2.1$ channels (Lee et al. 2003). Mutations of the IQ-like domain primarily impair facilitation, indicating that they interact primarily with the C-terminal lobe of CaM (DeMaria et al. 2001; Lee et al. 2003). In contrast, mutations of the CBD predominately impair Ca^{2+}-dependent inactivation (Lee et al. 2003), suggesting that they interact primarily with the lower affinity N-terminal lobe of CaM. These results lead to a model in which rapid, high-affinity binding of Ca^{2+} to the C-terminal lobe of CaM and interaction with the IQ-like motif of $Ca_V2.1$ channels cause facilitation, whereas subsequent slower and/or lower-affinity binding of Ca^{2+} to the N-terminal lobe of CaM and interaction with the CBD of $Ca_V2.1$ channels cause inactivation (Catterall and Few 2008).

1.3.8 Regulation of Ca^{2+} Channel by CaBP1 and VILIP-2

Calmodulin-like Ca^{2+} sensor proteins (CaS), that possess four EF-hand Ca^{2+}-binding motifs organized in two lobes connected by a central α helix, are expressed in neurons. CaBP1 is a member of a subfamily of neuron specific CaS (nCaS) highly expressed in the brain and retina (Haeseleer et al. 2000) and is colocalized with presynaptic $Ca_V2.1$ channels in some synapses (Lee et al. 2002). Like CaM, CaBP1 binds to the CBD of $Ca_V2.1$, but its binding is Ca^{2+} independent (Lee et al. 2002). CaBP1 causes rapid inactivation that is independent of Ca^{2+}, and it does not support Ca^{2+}-dependent facilitation (Lee et al. 2002). Another nCaS visinin-like protein-2 (VILIP-2) highly expressed in the neocortex and hippocampus (Burgoyne and Weiss 2001) also modulates $Ca_V2.1$ channels. VILIP-2 increases Ca^{2+}-dependent facilitation, but inhibits Ca^{2+}-dependent inactivation (Lautermilch et al. 2005). CBD and IQ-like motifs of $Ca_V2.1$ are required for binding of VILIP-2. Thus, CaBP-1 and VILIP-2 bind to the same site as CaM, but have opposite effects on $Ca_V2.1$ channel activity. In a presynaptic terminal, these differential effects on facilitation and inactivation of the P/Q-type Ca^{2+} current would substantially change the encoding properties of the synapse in response to trains of action potentials (Abbott and Regehr 2004). Why does different nCaS regulate $Ca_V2.1$ function at the same sites as CaM? Their affinity and binding speed to Ca^{2+} are different (Faas et al. 2011). CaM has lower affinity and higher binding speed to

Ca^{2+} than nCaS, suggesting temporal regulation of $Ca_V2.1$ activity by CaM and nCaS proteins. The divergent actions of nCaS proteins on $Ca_V2.1$ channels may fine-tune the function and regulatory properties of presynaptic P/Q-type Ca^{2+} currents, allowing a greater range of input-output relationships and short-term plasticity at different synapses (Catterall and Few 2008).

1.3.9 Regulation of Ca^{2+} Channel by CaMKII

Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is the most prominent Ca^{2+}/CaM-dependent regulator of the postsynaptic response, including long-term potentiation (Schulman and Greengard 1978; Kennedy et al. 1990; Luscher et al. 2000; Shepherd and Huganir 2007). CaMKII also regulates presynaptic function (Llinas et al. 1985, 1991), including effects on synaptic plasticity (Chapman et al. 1995; Lu and Hawkins 2006). CaMKII binds to $Ca_V2.1$ channel ($\alpha1_{1897–1912}$) and enhances activity by slowing inactivation and positively shifting the voltage dependence of inactivation (Jiang et al. 2008). Surprisingly, these effects on the function of $Ca_V2.1$ channels require binding of an autophosphorylated form of CaMKII (Magupalli et al. 2013), but do not require the catalytic activity of the enzyme (Jiang et al. 2008). Dephosphorylation of CaMKII does not reverse the binding (Magupalli et al. 2013). Furthermore, CaMKII binding to $Ca_V2.1$ C-terminus$_{1766–2212}$, increases phosphorylation of synapsin-1 and induces oligomers of synapsin-1 (Magupalli et al. 2013). It was proposed that noncatalytic regulation of $Ca_V2.1$ channels by bound CaMKII serves to enhance the activity of those channels that have the effector of the Ca^{2+} signal (i.e., CaMKII) in position to bind entering Ca^{2+} and respond to it (Jiang et al. 2008). This form of regulation is similar to regulation by SNARE proteins and RIM, as described above; that is, the activity of the $Ca_V2.1$ channels is increased by formation of a complete SNARE complex with synaptotagmin and RIM bound (Zhong et al. 1999; Kiyonaka et al. 2007), which serves as the effector of the Ca^{2+} signal for initiation of synaptic transmission. This ‘effector checkpoint’ mechanism serves to focus Ca^{2+} entry through those Ca^{2+} channels whose effectors (i.e., a complete SNARE complex and CaMKII) are bound and ready to respond (Jiang et al. 2008; Magupalli et al. 2013).

1.4 Ca^{2+} Channel and SNARE Protein Complex Regulates Synaptic Transmission

Peptides derived from the synprint site competitively inhibit interactions between SNARE proteins and Ca_V2 channels *in vitro*. Injection of synprint peptides from $Ca_V2.2$ channels into presynaptic SCG neurons in culture significantly reduced

the excitatory postsynaptic response by competitive uncoupling of the endogenous Ca^{2+} channel-SNARE interaction at nerve terminals (Mochida et al. 1996). Rapid, synchronous synaptic transmission was selectively inhibited following the injection, while late asynchronous release and paired-pulse facilitation were increased. Similarly, injection of the synprint peptides into embryonic Xenopus spinal neurons reduced transmitter release substantially when cells were stimulated in an extracellular solution containing physiological Ca^{2+} concentration (Rettig et al. 1997). Increasing the external Ca^{2+} concentration effectively rescued this inhibition, implying that the Ca^{2+} channels are competitively displaced away from docked synaptic vesicles by the injected synprint peptides, and this effect can be overcome by flooding the presynaptic terminal with Ca^{2+} from the extracellular pool (Rettig et al. 1997). A requirement for close coupling of $Ca_V2.1$ channels to synaptic vesicles for efficient release of neurotransmitters also emerged from studies at the calyx of Held. P/Q-type Ca^{2+} currents are more effective than N-type Ca^{2+} currents and R-type Ca^{2+} currents in eliciting neurotransmitter release at this synapse in postnatal day seven rats where all three channels are expressed (Wu et al. 1999; Iwasaki et al. 2000; Inchauspe et al. 2007). The high efficiency of P/Q-type Ca^{2+} currents in initiating neurotransmitter release is correlated with the close localization of docked vesicles near $Ca_V2.1$ channels (Wadel et al. 2007), as assessed by immunocytochemistry (Wu et al. 1999). At first glance, it seems that interactions of Ca_V2 channels with SNARE proteins have two opposing effects: tethering synaptic vesicles near the point of Ca^{2+} entry would increase synaptic transmission, whereas enhancing Ca_V2 channel inactivation would reduce synaptic transmission. These effects were dissected by use of competing synprint peptides and mutant syntaxin in *Xenopus* neuromuscular junctions *in vivo* (Keith et al. 2007). Injection of competing synprint peptides into developing neuromuscular junctions reduced the basal efficiency of synaptic transmission, as reflected in increased paired-pulse facilitation and reduced quantal content of synaptic transmission. Evidently, the effect of the synprint peptide to reduce linkage of docked synaptic vesicles to Ca_V2 channels is predominant, because its potentially opposing effect to relieve inhibition of Ca^{2+} channels by SNARE proteins would be occluded by SNAP-25 and synaptotagmin for the subset of channels interacting with a complete SNARE complex that could participate in vesicle release. In contrast, overexpression of a syntaxin mutant that is unable to regulate $Ca_V2.2$ channels, but still binds to them (Bezprozvanny et al. 2000), increased the efficiency of synaptic transmission, as reflected in reduced paired-pulse facilitation and increased quantal content (Keith et al. 2007). In this case, the syntaxin mutant likely relieves enhanced inactivation of $Ca_V2.2$ channels caused by endogenous syntaxin, thereby increasing Ca^{2+} entry and synaptic transmission, but does not alter linkage of docked synaptic vesicles to $Ca_V2.2$ channels. These results demonstrate a bidirectional regulation of synaptic transmission *in vivo* by interactions of SNARE proteins with $Ca_V2.2$ channels.

1.5 Ca^{2+} Channel and Gβγ protein Complex Regulates Synaptic Transmission

Potent negative regulation of neurotransmission by receptor activation is mediated by Gβγ modulation of presynaptic Ca_V2 channels in the CNS. At the calyx of Held this type of modulation by GABA acting at GABA-B receptors and glutamate acting at metabotropic glutamate receptors has been directly demonstrated with parallel measurements of Ca^{2+} currents and synaptic transmission (Takahashi et al. 1996; Kajikawa et al. 2001). Similar modulation by cannabinoids acting at CB1 receptors has been demonstrated by optical measurements of Ca^{2+} transients together with electrophysiological recordings of synaptic transmission at the nerve terminals of the parallel fibers of cerebellar granule cells innervating Purkinje neurons (Brown et al. 2004).

Depolarization relieves this form of inhibition of Ca^{2+} channels, leading to the prediction that trains of action potentials would reverse receptor/G protein inhibition of synaptic transmission. This prediction has been tested in hippocampal neurons in which autapses are formed by single hippocampal pyramidal neurons (Brody and Yue 2000). Trains of action potential-like stimuli relieve the inhibition of synaptic transmission caused by activation of GABA-B receptors. This relief of inhibition resulted in facilitation of synaptic transmission in the range of 1.5-fold, which was blocked by inhibition of $Ca_V2.1$ channels, but not $Ca_V2.2$ channels, with peptide neurotoxins. Regulator of G protein signaling-2 (RGS-2) relieves G protein inhibition of presynaptic Ca^{2+} channels, resulting in a higher basal probability of release and consequently a reduction in paired-pulse facilitation ratio (Han et al. 2006). These results demonstrate that voltage-dependent relief of G protein inhibition of Ca_V2 channels in paired-pulses and trains can cause synaptic facilitation. However, this form of facilitation does not contribute to short-term synaptic plasticity at parallel fibers synapses onto Purkinje cells (Kreitzer and Regehr 2000).

Gβγ caused potent negative regulation of neurotransmission by modulation of presynaptic $Ca_V2.2$ channels in SCG neurons (Stephens and Mochida 2005). Injection of purified Gβγ into presynaptic neurons reduced transmitter release. The Gβγ injected neurons cannot induce reduction of synaptic transmission with noradrenaline application. Noradrenaline shortens action potential duration by inhibition of Ca^{2+} current, resulting in reduction of transmitter release. Noradrenaline does not change the synaptic vesicle pool size (Stephens and Mochida 2005).

1.6 Ca^{2+} Channel Activity and Presynaptic Short-Term Plasticity

Short-term synaptic plasticity of neurotransmitter release from presynaptic terminals shapes the response of postsynaptic neurons to bursts of impulses and is crucial for fine-grained encoding of information in the nervous system (Zucker and Regehr

2002; Abbott and Regehr 2004). Regulation of presynaptic Ca^{2+} channels by Ca^{2+}, CaM, and nCaS proteins causes facilitation and inactivation of the Ca^{2+} current. The steep dependence of neurotransmitter release on the presynaptic Ca^{2+} current predicts that this type of regulation should profoundly alter short-term synaptic plasticity. Differential expression of these Ca^{2+}-dependent regulatory proteins may provide a means of cell-type-specific regulation of presynaptic Ca^{2+} channels and short-term synaptic plasticity.

1.6.1 Activity-Dependent $Ca_V2.1$ Channels Facilitation and Synaptic Enhancement

At the calyx of Held, presynaptic Ca^{2+} current can be recorded directly by voltage-clamp methods. In synapses from young mice, a combination of P/Q- and N-type currents shows activity-dependent facilitation that predicts the amount of synaptic facilitation according to the power law (Inchauspe et al. 2004; Ishikawa et al. 2005). In contrast, both facilitation of the presynaptic Ca^{2+} current and synaptic facilitation are lost in $Ca_V2.1$ knockout mice (Inchauspe et al. 2004, 2007; Ishikawa et al. 2005). The N-type Ca^{2+} currents conducted by $Ca_V2.2$ channels that remain in the calyx of Held of these $Ca_V2.1$ knockout mice are less efficient in mediating synaptic transmission, do not show facilitation, and do not support facilitation of synaptic transmission, but they are more sensitive to modulation by G protein-coupled receptors (Inchauspe et al. 2007). These results suggest that activity-dependent increases in presynaptic $Ca_V2.1$ channel currents cause synaptic facilitation and that $Ca_V2.2$ channel currents are not increased by facilitation, but have strong G protein regulation.

Presynaptic trains of action potentials generate augmentation and posttetanic potentiation (PTP) relying on residual Ca^{2+}. The relationship between presynaptic Ca^{2+} transients and PTP was measured at the calyx of Held using fluorescent Ca^{2+} indicators. After induction of PTP, the presynaptic Ca^{2+} influx increased to an extent that predicted PTP when the power law of neurotransmission was applied (Habets and Borst 2005). Furthermore, the presynaptic Ca^{2+} transient decayed with a time course that paralleled the decay of PTP (Habets and Borst 2006). These results are consistent with a role for regulation of presynaptic Ca^{2+} channels in PTP at the calyx of Held.

$Ca_V2.1$ channel modulation may be involved in presynaptic facilitation of the calyx of Held and other synapses. To study function of presynaptic $Ca_V2.1$ channels, synapses that express homogeneous $Ca_V2.1$ channels are ideal. SCG neurons have endogenous N-type but not P/Q-type Ca^{2+} currents. In the presence of N-type Ca^{2+} blocker, ω-conotoxin GVIA, P/Q-type Ca^{2+} currents are recorded from neurons transfected with cDNA encoding $Ca_V2.1$ channels by microinjection (Mochida et al. 2003a, 2008). Whole-cell voltage-clamp recordings of transfected $Ca_V2.1$ channels at the cell body show that they undergo Ca^{2+}-dependent facilitation

(Mochida et al. 2008). In these transfected SCG neurons, mutations in the IQ-like motif of $Ca_V2.1$ channels that prevent Ca^{2+}-dependent facilitation of Ca^{2+} currents recorded from the cell bodies of SCG neurons, also reduced paired-pulse facilitation and augmentation of EPSPs at SCG synapses (Mochida et al. 2008). In addition, expression of VILIP-2, which blocks Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+} current, induced paired-pulse facilitation. Ca^{2+} concentration-dependent synaptic facilitation was also induced during action potential trains. However, VILIP-2 did not induce paired-pulse and synaptic facilitation in the neuron expressed with IMCBD mutant $Ca_V2.1$ channels (Leal et al. 2012). Thus, CaM and VILIP-2 may respond to residual Ca^{2+} as 'facilitation sensors' by binding to the IQ-like motif in the C terminus of $Ca_V2.1$ channels and causing Ca^{2+}-dependent facilitation of the presynaptic Ca^{2+} current. In contrast, PTP induced by longer trains of stimuli was not significantly affected (Mochida et al. 2008). Thus, synaptic facilitation and augmentation in transfected SCG neurons share a common mechanism: activation of nCaS proteins by residual Ca^{2+} increases 'instantaneous' Ca^{2+} entry via $Ca_V2.1$ channels in an activity-dependent manner, which in turn increases neurotransmitter release according to the power law of neurotransmission. This increase in Ca^{2+} entry *via* $Ca_V2.1$ channels directly mediates multiple forms of synaptic enhancement—facilitation, augmentation, and perhaps PTP in some synapses—by increasing neurotransmitter release according to the power law. While facilitation of presynaptic Ca^{2+} channels may contribute to all three forms of synaptic enhancement at some synapses (Ishikawa et al. 2005; Mochida et al. 2008), augmentation and PTP likely represent overlapping processes that are caused by different combinations of mechanisms at different synapses (Zucker and Regehr 2002). Expression of $Ca_V\beta$ subunits has a strong influence on synaptic facilitation in hippocampal synapses through their effects on Ca^{2+} channel function (Xie et al. 2007).

1.6.2 Activity-Dependent $Ca_V2.1$ Channels Inhibition and Synaptic Depression

Synaptic depression is generally thought to be a result of vesicle depletion during trains of action potentials (Zucker and Regehr 2002). At the calyx of Held, stimulation at 100 Hz induced robust synaptic depression that was likely caused by vesicle depletion (Xu and Wu 2005). In a prominent feature of synaptic transmission, the depression is caused by a decrease in release probability (Wu and Borst 1999). Ca^{2+}-dependent inactivation of the presynaptic Ca^{2+} current, rather than vesicle depletion, causes rapid synaptic depression for stimuli ranging from 2 to 30 Hz (Forsythe et al. 1998; Xu and Wu 2005). Introduction of peptides that disrupt CaM interactions reduced both Ca^{2+}-dependent inactivation of the P/Q-type Ca^{2+} current and paired-pulse depression of synaptic transmission (Xu and Wu 2005).

In transfected SCG neurons (Mochida et al. 2008), deletion of the CaM-binding domain (CBD) in the intracellular C terminus of full-length $Ca_V2.1$ channels, a mutation known to reduce Ca^{2+}-dependent inactivation in heterologous expression systems (Lee et al. 1999, 2003), blocked paired-pulse depression and reduced synaptic depression during trains up to 40 Hz (Mochida et al. 2008). In addition, expression of CaBP1, which blocks Ca^{2+}-dependent facilitation of P/Q-type Ca^{2+} current, markedly induced paired-pulse depression and synaptic depression during trains. However, CaBP1 did not show synaptic depression in the neuron expressed with IMCBD mutant $Ca_V2.1$ channels (Leal et al. 2012). These results suggest that binding of CaM and CaBP1 to the CBD induce inactivation of presynaptic $Ca_V2.1$ channels, resulting in rapid synaptic depression. During trains at 30 Hz and 40 Hz, a slower phase of synaptic depression was observed that may have been caused by vesicle depletion. Together, the data from the calyx of Held and transfected SCG neurons suggest that Ca^{2+}-dependent inactivation of presynaptic Ca^{2+} channels, mediated by Ca^{2+}-dependent binding of CaS proteins to the C-terminal of Ca_V2 channels, is a conserved mechanism generating rapid synaptic depression evoked by stimuli of physiological rate and duration (at 40 Hz for 1 s) at multiple synapses. Results of studies with cultured hippocampal neurons also support an important role for modulation of Ca_V2 channels in synaptic plasticity. Overexpression of $Ca_V\beta4$ favors facilitation whereas overexpression of $Ca_V\beta2$ favors depression (Xie et al. 2007).

1.6.3 CaMKII Regulates Short-Term Synaptic Plasticity

Binding of CaMKII to $Ca_V2.1$ channels enhances their functional activity by inhibiting their inactivation (Jiang et al. 2008) and enhances the activity of CaMKII by increasing its autophosphorylation (Magupalli et al. 2013). In order to critically test the potential effects of this specific interaction on synaptic transmission, it is necessary to manipulate the activity of CaMKII bound specifically to $Ca_V2.1$ channels in the presynaptic terminal without altering the functional activity of CaMKII in the postsynaptic compartment or CaMKII in other locations in the presynaptic terminal. Accordingly, we expressed $Ca_V2.1$ channels in SCG neurons in culture. Paired-pulse facilitation of synaptic transmission in this transfected SCG neuron is primarily caused by facilitation of $Ca_V2.1$ channel activity by Ca^{2+}/CaM and CaS protein binding to the IQ-like domain in the C-terminus (Mochida et al. 2008; Leal et al. 2012). As illustrated in Fig. 1.2, $Ca_V2.1$ channels expressed alone generate synaptic transmission in which the paired-pulse ratio is highly dependent on the inter-stimulus interval (ISI) between the paired pulses. At short ISI, synaptic depression is dominant and paired-pulse ratio values are less than 1.0. At longer ISI, synaptic facilitation becomes dominant, peaks at ~1.75 for an ISI of 80 ms, and declines to 1.0 at long ISI (Fig. 1.2). Perfusion of a competing peptide that

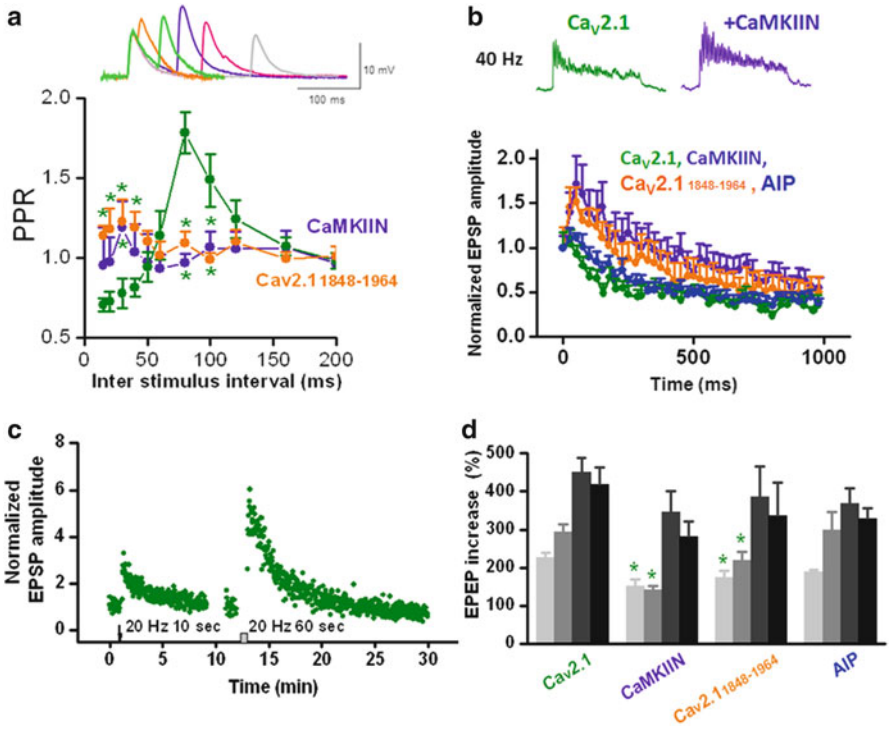

Fig. 1.2 CaMKII dysfunction prevented presynaptic facilitation and depression mediated by transfected $Ca_V2.1$ channel facilitation and inactivation in cultured superior cervical ganglion (SCG) neurons. (**a**) Inter-stimulus interval dependent paired-pulse depression (PPD) and facilitation (PPF) (*upper traces*). Both PPD and PPF are prevented by CaMKII inhibitory peptide (CaMKIIN) or CaMKII-binding site peptide of $Ca_V2.1$ channel (Adapted from Magupalli et al. 2013). (**b**) Synaptic depression during a 2-s train of action potentials (at 40 Hz) changed to synaptic facilitation in the presence of CaMKIIN (*upper traces*) or CaMKII-binding site peptide of $Ca_V2.1$ channel but not a CaMKII phosphorylation competitor, AIP. (**c**) A 10-s and 60-s trains of action potentials at 20 Hz in the $Ca_V2.1$-transfected presynaptic neurons induce augmentation and PTP, respectively. Graph shows normalized amplitudes of EPSPs recorded every 2 s. (**d**) Augmentation induced by 10-s trains at 20 Hz (*light grey bar*) and at 40 Hz (*grey bar*) was reduced by CaMKIIN and CaMKII-binding site peptide of $Ca_V2.1$ channel. In contrast, PTP induced by 60-s trains at 20 Hz (*dark grey bar*) and at 40 Hz (*black bar*) was not reduced by CaMKIIN and CaMKII-binding site peptide of $Ca_V2.1$ channel. These results suggest that CaMKII-mediated Ca channel facilitation is responsible for augmentation but not PTP, consistent with effect of IM-AA mutation that prevents augmentation but not PTP

blocks the interaction of CaMKII with $Ca_V2.1$ channels ($Ca_V2.1_{1848–1964}$) prevented both paired-pulse facilitation and paired-pulse depression at this model synapse (Fig. 1.2), suggesting that binding of CaMKII to $Ca_V2.1$ channels is required for expression of this regulatory effect. Similarly, expression of the brain-specific CaMKII inhibitor CaMKIIN (Chang et al. 1998), which prevents CaMKII binding

to $Ca_V2.1$ channels (Jiang et al. 2008), also prevented paired-pulse facilitation (Fig. 1.2). $Ca_V2.1_{1848–1964}$ or CaMKIIN also prevented synaptic depression during a train of action potentials and augmentation after a conditioning train (Fig. 1.2). It is unlikely that the basal release probability is affected by competing peptide injection or CaMKIIN expression because the mean amplitudes of the first EPSPs are unchanged. Evidently, binding of CaMKII by $Ca_V2.1$ channels is required for both up-regulation of channel activity in paired-pulses and for Ca^{2+}-independent activation of CaMKII by $Ca_V2.1$, and one or both of these effects is necessary for normal short-term synaptic plasticity.

Voltage-gated Ca^{2+} channels are regulated by their effectors such that the channels are more active when the effectors of their Ca^{2+} signal are bound. Examples include regulation of the skeletal muscle Ca^{2+} channel by the ryanodine-sensitive Ca^{2+} release channel (Nakai et al. 1996), its effector in excitation-contraction coupling, and regulation of presynaptic Ca^{2+} channels by SNARE proteins, which are the effectors for Ca^{2+}-dependent exocytosis (Catterall and Few 2008). Regulation of $Ca_V2.1$ channels by CaMKII also fits this regulatory theme (Jiang et al. 2008). Binding of CaMKII to $Ca_V2.1$ increases the activity of both binding partners, and their interaction is required for facilitation of synaptic transmission and perhaps for other aspects of presynaptic function. Enhancement of the activity of Ca^{2+} channels whose effectors are bound would focus Ca^{2+} entry and Ca^{2+}-dependent protein phosphorylation in locations where it can effectively generate a cellular response *via* local Ca^{2+} signaling. This mechanism would enhanced local signal transduction and reduce ineffective Ca^{2+} entry and protein phosphorylation at other sites (Magupalli et al. 2013).

1.7 Conclusion

Modulation of presynaptic Ca^{2+} channels has a powerful influence on synaptic transmission. Activation of autoreceptors and retrograde signaling receptors couples G-protein-mediated tonic inhibition of N-type Ca^{2+} channels activity that can be controlled by synaptic firing. At the pre firing state, activity of Ca^{2+} channels is inhibited by interaction with synaptic proteins at the active zone. The inhibition is relieved by switching to interact with SNAREs and synaptotagmin, which are the effectors for Ca^{2+}-dependent exocytosis. During and post firing, activity of Ca^{2+} channels is regulated by residual Ca^{2+} that is caught temporally by CaM and nCaS proteins with individual Ca^{2+} affinity and Ca^{2+} binding speed. CaMKII binding to $Ca_V2.1$ is a spatial effector that increases the activity of both binding partners and their interaction is required for facilitation and depression of synaptic transmission (Fig. 1.3). Fine-tuning the function and regulatory properties of presynaptic P/Q-type Ca^{2+} currents allow a greater range of input-output relationships and short-term plasticity.

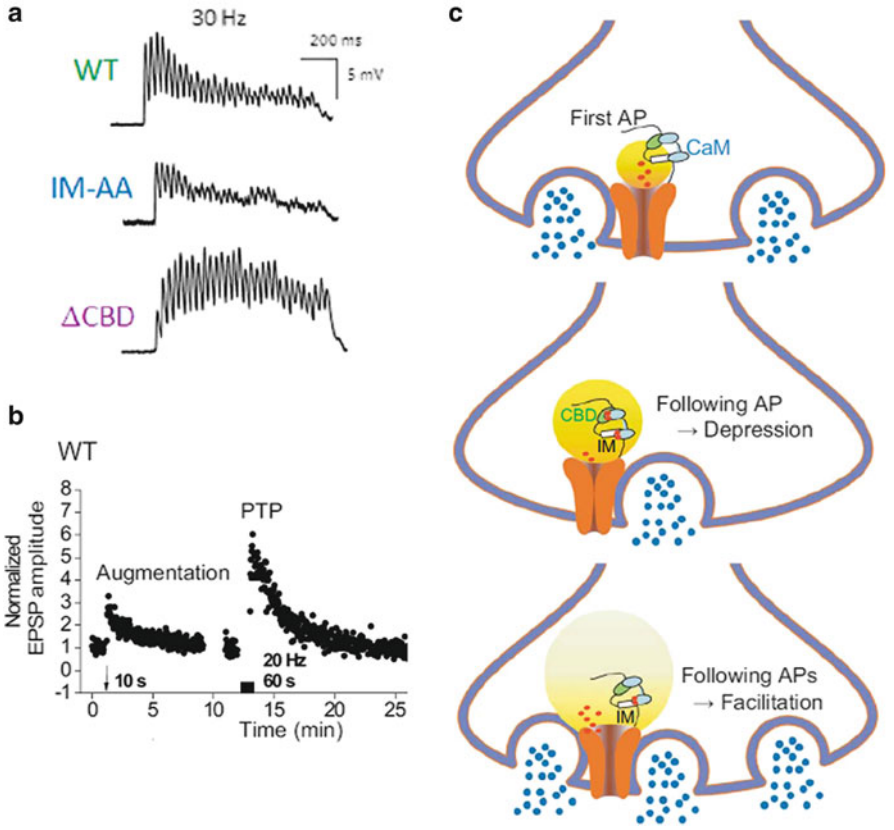

Fig. 1.3 Presynaptic facilitation and depression mediated by $Ca_V2.1$ channel facilitation and inactivation. (**a**) Averaged trace of EPSPs (n = 5–12), in which $Ca_V2.1$ channels were the only active channels in the presence of ω-conotoxin GVIA, evoked by action potentials with 1 s train at 30 Hz in SCG neuron in culture. WT shows synaptic facilitation and depression. IM-AA mutant shows presynaptic depression, while CBD shows synaptic facilitation. These results suggest that IM is responsible for presynaptic facilitation and that CBD is responsible for presynaptic depression (Adapted from Mochida et al. 2008). (**b**) Normalized and averaged amplitudes of EPSPs recorded every 2 s from SCG neuron synapses in the presence of ω-conotoxin GVIA. Conditioning stimuli were applied at the indicated times at 20 Hz for 10 s to evoke augmentation and at 20 Hz for 60 s to induce PTP (Adapted from Mochida et al. 2008). (**c**) Model illustrating $Ca_V2.1$-mediated mechanisms of synaptic depression, and facilitation and augmentation. In synaptic depression, CaM sensing local Ca^{2+} interacts with the CaM-binding domain (CBD) to cause channel inactivation and reduce Ca^{2+} entry, thus reducing neurotransmitter release. In synaptic facilitation and augmentation, CaM sensing global Ca^{2+} interacts with the IQ-like motif to cause channel facilitation and increase in Ca^{2+} entry and subsequently neurotransmitter release increases. nCaS proteins bind to the IM and the CBD to induce synaptic facilitation and depression (Adapted from Mochida 2011)

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Part II
Interaction Partners of Calcium Channels

Chapter 2
Neuronal Functions of Auxiliary Calcium Channel Subunits

Gerald J. Obermair and Bernhard E. Flucher

Abstract In the central nervous system the second messenger calcium regulates neurotransmitter release, gene regulation, and neuronal plasticity. Voltage-gated calcium channels provide the major regulated calcium entry pathway in the membrane of neurons. They operate in a heteromultimeric complex between a pore forming α_1, and the auxiliary β and $\alpha_2\delta$ subunits. The cytoplasmic β and the extracellular membrane-attached $\alpha_2\delta$ subunit are required for the proper functional expression of the entire calcium channel complex. Moreover, the auxiliary subunits modulate the gating properties of the calcium channel and serve as scaffolds for upstream regulators and downstream effectors. Any of these properties affect the size of the calcium signal and in the synapse lead to changes in the functional coupling to neurotransmitter release. Beyond their classical role as auxiliary calcium channel subunits, β and $\alpha_2\delta$ have recently been implicated in cellular and neuronal functions independent of the channel complex. Here we review the experimental evidence pertinent to the many facets of auxiliary calcium channel function. We extract from it common principles and attempt to depict the state of the art of their role in regulating presynaptic function.

Keywords Voltage-gated calcium channels • Synaptic transmission • $\alpha_2\delta$ • β • High-voltage activated Ca^{2+} channels • Channel trafficking

2.1 Introduction

In excitable cells voltage-gated calcium channels (Ca_Vs; also termed voltage-dependent or voltage-operated calcium channels) mediate and regulate a variety of functions ranging from muscle contraction, secretion, synaptic function to gene

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regulation. Calcium entering through voltage-gated calcium channels operates as a local second messenger by activating downstream signalling proteins localized in the close vicinity of the channel pore. In neurons Ca_Vs contribute to the specific action potential firing pattern, presynaptic Ca_Vs regulate neurotransmitter release (Stanley 1993) and postsynaptic Ca_Vs are involved in the transcriptional regulation of CREB (cAMP-responsive element-binding protein) and NFAT (nuclear factor of activated T cells) (Deisseroth et al. 2003; Dolmetsch 2003) and thus likely play a crucial part in the formation of new memory (Moosmang et al. 2005). Over the recent years a detailed picture on the distribution and function of pre- and postsynaptic calcium channel types has begun to emerge. Thus, in the central nervous system Ca_Vs of the Ca_V2 family, namely P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and R-type ($Ca_V2.3$) channels, are the major presynaptic pore forming subunits triggering synaptic release. The L-type channels $Ca_V1.2$ and $Ca_V1.3$ are mainly involved in postsynaptic functions including plasticity and gene transcription. The importance of the pre- and postsynaptic Ca_V pore-forming subunits is emphasized by the existence of channelopathies caused by loss-of-function as well as gain-of-function mutations (Pietrobon 2010; Striessnig et al. 2010). For example, dysregulation of presynaptic P/Q-type and postsynaptic L-type channels is involved in the etiology of migraine (Pietrobon 2010) and autism disorders (Splawski et al. 2004), respectively. In contrast, there is little to no evidence for a function of the skeletal muscle $Ca_V1.1$ isoform in the nervous system (Sinnegger-Brauns et al. 2009). The $Ca_V1.4$ isoform appears to be specifically expressed in the retina and its mutation causes congenital stationary night blindness type 2 (Wycisk et al. 2006a, b). Low-voltage activated calcium channels (T-type channels) $Ca_V3.1$, 3.2 and 3.3 are critical regulators of neuronal excitability. They are prominently expressed both in the central and peripheral nervous system and are involved in neurological disorders such as absence epilepsy and neuropathic pain (Iftinca 2011).

Ca_Vs operate in heteromultimeric complexes with the auxiliary β (also termed $Ca_V\beta$) and $\alpha_2\delta$ subunits, calmodulin and other calcium binding and regulating proteins. The pore-forming α_1 subunit of voltage-gated calcium channels defines the basic biophysical, pharmacological and physiological properties of the channels. A plethora of studies within the last 20 years have extensively demonstrated their roles in the localization, trafficking and stabilization of the channel complex (reviewed in Arikkath and Campbell 2003; Obermair et al. 2008; Dolphin 2009; Buraei and Yang 2010). The great majority of these studies was performed with different channel subunit combinations heterologously expressed in Xenopus laevis oocytes or mammalian expression systems such as human embryonic kidney (HEK) cells. Therefore the informative value of these studies regarding the role of the auxiliary calcium channel subunits in native cell systems like neurons remained limited. Whereas studies in heterologous expression systems are ideally suited to investigate effects and mechanisms for the interaction of specific coexpressed subunit partners in isolation, such studies do not predict as to whether the same protein-protein interactions indeed occur in signaling complexes of differentiated cells. Neither can it be assumed that in the complex with additional up- and downstream interacting proteins in differentiated cells the properties and effects

of such interactions are the same as in heterologous expression systems. The development of powerful neuronal expression systems and the analysis of calcium channel knock-out animal models (see box) in recent years have helped to reveal the physiological importance of auxiliary β and $\alpha_2\delta$ subunits in neuronal/synaptic function. With respect to the role of auxiliary calcium channel subunits in synaptic function the principal questions that now can be addressed include:

- What is the complement of specific calcium channel isoforms expressed in synaptic compartments?
- Do different subunit isoforms serve distinct functions and to what degree can they be compensated by other isoforms?
- Do the auxiliary subunits exclusively function in the context of the calcium channel (i.e., regulate its expression and targeting, or modulate its gating properties) or do auxiliary calcium channel subunits also serve functions independent of the channel?

2.2 Structure and Function of Auxiliary Calcium Channel Subunits

2.2.1 The $\alpha_2\delta$ Subunit

A total of four genes (Cacna2d1-4) encode for $\alpha_2\delta$ subunits ($\alpha_2\delta$-1 to $\alpha_2\delta$-4), which display distinct tissue distribution and out of which three isoforms ($\alpha_2\delta$-1 to -3) are strongly expressed in the central nervous system (CNS) (Arikkath and Campbell 2003; Schlick et al. 2010). $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits are the primary targets for the anti-epileptic and anti-allodynic drugs gabapentin (GBP) and pregabalin (PG), which have also proven clinical efficacy in the treatment of generalized anxiety disorders (Bryans and Wustrow 1999; Rickels et al. 2005). Mature $\alpha_2\delta$ subunits consist of posttranslationally cleaved α_2 and δ peptides, which are associated to each other by a disulfide bond (Calderon-Rivera et al. 2012). Until recently it had been suggested that the δ subunit constitutes a single-pass membrane protein, and the α_2 subunit a highly glycosylated extracellular protein. However, this classical view has recently been challenged by the observation that $\alpha_2\delta$ subunits can form GPI-anchored proteins and that this posttranslational modification may be crucial for $\alpha_2\delta$ function (Davies et al. 2010). In either way the vast majority of the $\alpha_2\delta$ protein is extracellular, ideally situated to interact with constituents of the extracellular matrix or extracellularly exposed proteins. Consistent with a role in extracellular signaling is the domain structure of α_2. A von Willebrand factor type A (VWA) domain and two Cache domains were identified by sequence homology in all $\alpha_2\delta$ subunits (Anantharaman and Aravind 2000; Canti et al. 2005; Davies et al. 2007). VWA-domains are found in a variety of extracellular matrix proteins and integrin receptors and are well known for their role in cell-cell adhesion (Whittaker and Hynes 2002) involving a metal ion-dependent adhesion site (MIDAS). The integrity

of the MIDAS motif in $\alpha_2\delta$-2 has been shown to be necessary for calcium current enhancement and Ca_V channel trafficking (Canti et al. 2005). Cache domains were named after their presence in calcium channels and chemotaxis receptors and have been suggested to be involved in small molecule interactions (Anantharaman and Aravind 2000). Thus, it has been hypothesized that these domains may be regulated by small endogenous ligands, such as the amino acid isoleucine (reviewed in Dooley et al. 2007), and that they are involved in GBP and PG binding (Davies et al. 2007). $\alpha_2\delta$ subunits also contain a conserved N-terminal α-helical domain found in several methyl-accepting chemotactic receptors and mutations within this domain have been shown to interfere with GBP and PG binding (Anantharaman and Aravind 2000).

2.2.2 The β Subunit

The entirely cytoplasmic β subunit consists of a conserved SH3 protein interaction domain and a nucleotide kinase-like domain (Chen et al. 2004; Opatowsky et al. 2004; Van Petegem et al. 2004) and thus resembles in structure the membrane-associated guanylate kinase proteins (Dolphin 2003; Takahashi et al. 2005). However, the SH3 domain of β subunits differs from that of canonical polyprolin-binding pockets and the guanylate kinase fold is modified so that it lacks kinase activity. Instead it binds the intracellular I-II linker of α_1 subunits at the so-called α interaction domain (AID) with nanomolar affinity (De Waard et al. 1995; Van Petegem et al. 2008). The SH3 and the GK-like domains are highly conserved among the four genes encoding β subunits (Cacnb1-b4). The sequences connecting these domains and the N- and C-termini vary between isoforms and are subject to alternative splicing (Colecraft et al. 2002; Dolphin 2003). In the channel complex β subunits serve two roles: They have a chaperon function regulating the export of the calcium channel from the endoplasmic reticulum and thus membrane expression of functional channels (Fang and Colecraft 2011). Moreover, they modulate gating properties of the channel directly as well as by interaction with other regulatory proteins like Rab binding proteins or G-proteins. β itself is subject to PKA mediated phosphorylation (reviewed in Buraei and Yang 2010). The β_{2a} isoform is palmitoylated at two N-terminal cysteines and therefore membrane-associated even in the absence of an α_1 subunit. Nevertheless, the association of β subunits with the channel complex entirely depends on their binding to the AID in the α_1 subunit. This binding site in the cytoplasmic loop between repeats I and II of the α_1 subunit is a unique feature of the Ca_V1 and Ca_V2 subclasses of Ca_Vs. Accordingly, at least in heterologous expression systems all β subunits can associate with any of the Ca_V1 or Ca_V2 members. However, the low-voltage activated calcium channels of the Ca_V3 subclass do not associate with β subunits (Dolphin 2003). Because of their central role in regulating functional expression and biophysical properties of calcium channels, and because of the well defined interaction site, interfering with the AID-β interaction is an attractive strategy for designing specific calcium channel antagonists. So far, such endeavors

have not been successful. However, the high efficacy of members of the small G-protein Rem/Gem/Kir family in blocking calcium currents by interacting with the β subunit holds great promise for these calcium channel subunits as drug targets (Yang et al. 2007).

2.3 Neuronal Voltage-Gated Calcium Channel Complexes

In differentiated cells calcium channels do not function in isolation, rather they exert their functions in the context of multimolecular signalling complexes. The short range of the second messenger calcium necessitates that downstream signalling proteins and effectors are anchored in the close vicinity of the channel pore. Similarly, increasing evidence shows that upstream modulators, like protein kinases and phosphatases achieve substrate specificity and increased signalling efficiency if they pre-exist in a complex with the channel. Accordingly, a voltage-gated calcium channel complex is composed of the calcium channel subunits proper, upstream modulators and downstream effectors, and the adapter and scaffold proteins, assembling the complex.

In neurons two such complexes have been subject to extensive investigations: First, the synaptic vesicle fusion apparatus and second, the postsynaptic calcium channel complex mediating excitation-transcription coupling.

The presynaptic calcium channel complex: Calcium influx through Ca_Vs transduces membrane depolarization into the chemical signal triggering fusion of neurotransmitter vesicles. Here Ca_Vs of the Ca_V2 subclass (P/Q- and N-type) associate with the SNARE proteins of the synaptic core complex either directly by an interaction of the SYNPRINT domain within the II-III loop of the α_1 subunits with syntaxin, SNAP-25 and synaptotagmin-1 (reviewed in Sheng et al. 1998; Zamponi 2003; Catterall 2011), or via the β subunit and the Rab interacting protein (RIM) (Kiyonaka et al. 2007). These interactions are believed to anchor the Ca_V channel close to the calcium sensor synaptotagmin and conversely to functionally modulate the calcium current, both enhancing the channel's efficacy to activate vesicle fusion. Indeed a low number of channels and in the extreme even a single channel opening is sufficient for triggering vesicle fusion (Stanley 1993; Bucurenciu et al. 2010). Neurotransmitter release is commonly modulated by neuropeptides and hormones. Therefore G-protein coupled receptors (GPCRs), G-proteins, phospholipases, adenylate cyclases, and protein kinases may coexist with presynaptic calcium channel complexes.

The postsynaptic calcium channel complex: The postsynaptic Ca_V complex mediates excitation-transcription coupling. Here activation of L-type channels (Ca_V1) initiates a signalling cascade to the nucleus that regulates gene expression. To this end scaffold proteins like AKAP79/150 recruit protein kinases and the calcium/calmodulin dependent protein phosphatase calcineurin to the channel.

Upon activation by the local calcium signal this signalling cascade leads to the translocation of NFATc4 into the nucleus and (Oliveria et al. 2007; Ma et al. 2011). Since overexpression of AKAP79/150 also enhances the L-type calcium currents (Altier et al. 2002) it seems that at least some of these signalling proteins are shared with upstream signalling cascades mediating GPCR-induced phosphorylation of the channel. Indeed β adrenergic receptors, AKAP, PKA and calcineurin were all detected in the $Ca_V1.2$ signaling complex in neurons (Davare et al. 2001; Dai et al. 2009).

The subunit composition of the pre- and postsynaptic Ca_Vs is expected to influence the function of these signalling complexes in several ways. Modulation of current properties by auxiliary subunits will affect the signalling power of the complex. A participation of the auxiliary calcium channel subunits in scaffolding will affect the composition of the signalling complex and thus the signalling specificity. By targeting the channel into the close proximity of effector proteins the efficacy of the signalling process will be enhanced. As different subunits differ with respect to their modulatory properties, protein-protein interactions and subcellular targeting, as well as the molecular diversity of the auxiliary subunits may be important for the proper assembly and function of the different calcium channel complexes in neurons. In other words, the distinct molecular organizations and functions of different calcium channel signalling complexes may require a specific subunit composition of the channel. In turn, the distinct molecular compositions of pre- and postsynaptic signalling complexes may favour the incorporation of channels with specific subunit compositions.

2.3.1 Potential Mechanisms for Establishing Specific Neuronal Ca_V Complexes

Understanding the specificity of Ca_V subunit interactions in native differentiated cell systems is key for resolving the physiological neuronal functions of calcium channels and their auxiliary subunits. Three distinct mechanisms may explain the assembly of specific $\alpha_1/\beta/\alpha_2\delta$ subunit complexes in neurons (see Fig. 2.1):

1. Different affinities of auxiliary β and $\alpha_2\delta$ subunits for specific α_1 isoforms may favour the preferential association of specific subunit combinations.
2. Limiting the number of isoforms expressed in a specific cell type at a given time will also favour the formation of a specific Ca_V complex.
3. Distinct subcellular targeting properties of individual subunits as well as protein-protein interactions with other proteins may yield complex specificity.

Alternatively, specific stable complexes may not exist in all neuronal compartments and Ca_V channels could be regulated by reversible interactions with pools of functionally distinct cytoplasmic β subunits or membrane anchored $\alpha_2\delta$ subunits.

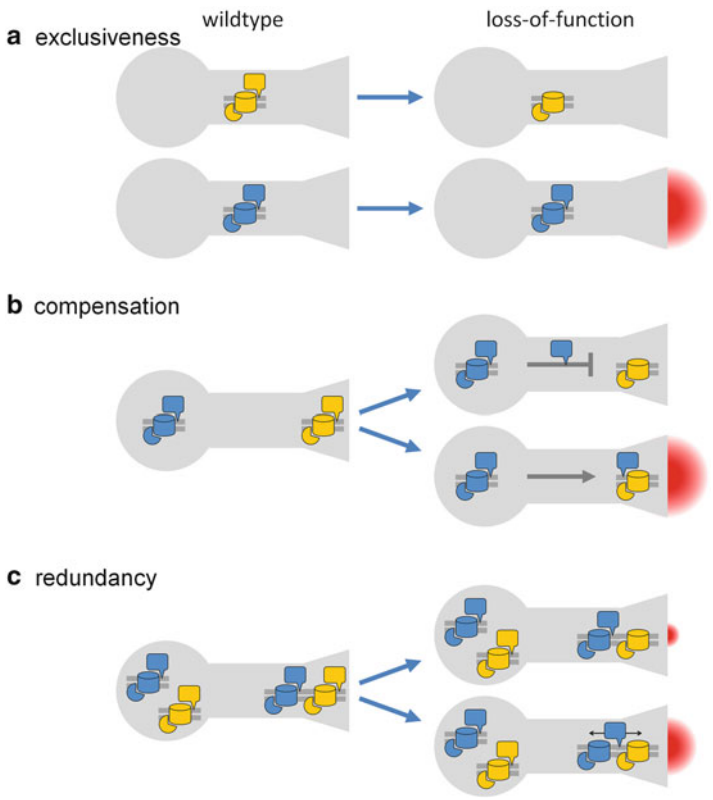

Fig. 2.1 Model explaining loss-of-function scenarios of distinct mechanisms for the assembly of specific $\alpha_1/\beta/\alpha_2\delta$ subunit complexes in neurons. (**a**) Different cell types may express exclusive $\alpha_1/\beta/\alpha_2\delta$ complexes (either *orange* or *blue*). Knockout of one subunit (*orange* $\alpha_2\delta$) will lead to a loss of function (e.g. synaptic function) in one cell type, whereas the functions of other cell types are not affected. (**b**) Specific $\alpha_1/\beta/\alpha_2\delta$ complexes may form by differential targeting into distinct subcellular compartments (soma-*blue*, synapse-*orange*). The consequence of knockdown of one subunit (*orange* $\alpha_2\delta$) depends on the uniqueness of the targeting properties of the remaining isoforms. Thus, if axonal targeting of the blue isoform is not possible, knockout will ultimately lead to a loss of function (*upper*). Otherwise the blue isoform may compensate for the loss of the orange isoform and restore normal synaptic function (*lower*). (**c**) Specificity of $\alpha_1/\beta/\alpha_2\delta$ complexes may be determined by distinct affinities or distinct interaction partners in a macromolecular complex. Putative compensatory mechanisms will then depend on the stringency of the individual complexes. If subunit exchange between the individual complexes is excluded, functional compensation is limited (*upper*). If subunits can associate/dissociate with and from individual complexes, at least partial compensation will occur (*lower*)

2.3.1.1 The Importance of Affinity for Specific Ca_V Complex Formation

Heterologous coexpression studies have demonstrated that all four β subunits as well as all four $\alpha_2\delta$ subunits can enhance the trafficking and modulate the current properties of all high-voltage activated calcium channel α_1 subunits, indicating a

great promiscuity of subunit interactions (reviewed in Arikkath and Campbell 2003; Dolphin 2003; Obermair et al. 2008; Buraei and Yang 2010). In line with this observation low neuronal α_1-β selectivity was suggested by immunoprecipitation experiments showing similar β subunit compositions of neuronal L-type, P/Q-type and N-type channels (Liu et al. 1996; Scott et al. 1996; Pichler et al. 1997). Furthermore, biochemical analysis revealed similarly high affinities of different β subunits to the AID (De Waard et al. 1995; Van Petegem et al. 2008). Therefore, if differences in the strength of interactions contribute to the formation of preferential subunit compositions, these may be determined by low affinity secondary interaction sites rather than by the AID, and/or by indirect interactions involving additional components of the signalling complex. In fact, recent experiments in skeletal muscle indicate that, although non-muscle β subunits can successfully compete with the skeletal muscle β_{1a} for association with the channel, complexes including the heterologous isoforms are less stable than those consisting of all skeletal muscle isoforms (Campiglio et al. 2013). These experiments for the first time demonstrate the formation of complexes with preferential subunit composition in a native calcium channel complex, and suggest that isoform specific differences in the association with the α_1 subunit underlie the distinct complex stabilities. For $\alpha_2\delta$ subunits no high-affinity binding sites have been identified in the α_1 subunit. Interestingly, although in the initial biochemical purification of neuronal Ca_V channels $\alpha_2\delta$ reliably co-purified with the α_1 and β subunits (McEnery et al. 1991; Witcher et al. 1994; Martin-Moutot et al. 1995; Liu et al. 1996), a recent quantitative proteomics approach of mammalian Ca_V2 channel complexes in brain extracts did not identify $\alpha_2\delta$ subunits as a core components of the complex (Müller et al. 2010).

Also upon coexpression in nerve or muscle cells, $\alpha_2\delta$ subunits appear more widely expressed throughout the plasma membrane than the other channel subunits (Schredelseker et al. 2005) (Schöpf, Obermair et al., unpublished observation). If distinct affinities of auxiliary subunit isoforms to preferential α_1 subunit partners contribute to the formation of specific complexes, this may involve low-affinity binding sites, which so far eluded biochemical detection. Such low affinity interaction would allow dynamic exchange of subunits in response to changes in expression levels or local concentration of the auxiliary subunits.

2.3.1.2 Spatial and Temporal Separation of Ca_V Subunit Expression

Distinct cellular expression levels of calcium channel isoforms indeed provide an important determinant of complex specificity. They have been identified in non-neuronal cells such as skeletal muscle ($Ca_V1.1/\beta_{1a}/\alpha_2\delta$-1) and cardiac myocytes ($Ca_V1.2/\beta_2/\alpha_2\delta$-1) (Arikkath and Campbell 2003) and specialized neuronal cell types like retina photoreceptor cells ($Ca_V1.4/\beta_2/\alpha_2\delta$-4) (Ball et al. 2002; Barnes and Kelly 2002; Wycisk et al. 2006a; Neef et al. 2009). Similarly, the cerebellum shows a strong preference towards expression of one subunit combination ($Ca_V2.1/\beta_4/\alpha_2\delta$-2) (Ludwig et al. 1997; Brodbeck et al. 2002). We have recently shown that murine

cortex, hippocampus and cerebellum simultaneously express mRNA of five out of seven high-voltage activated α_1 subunits, all four β subunit isoforms, and three of four $\alpha_2\delta$ subunits at physiologically relevant levels (Schlick et al. 2010). Surprising was our finding that also a single cell type, cultured hippocampal pyramidal cells expresses all the same Ca_V subunit isoforms as hippocampus. This clearly suggests that in cultured hippocampal pyramidal cells, a restricted expression of auxiliary subunit isoforms is not the strategy to achieve specific Ca_V subunit compositions. Consequently other mechanisms, like specific targeting properties and interactions with anchoring proteins in pre- and postsynaptic compartments, must be responsible for assembling channels with distinct subunit compositions.

In neurons expression patterns of channels and signaling proteins are not static. Many receptors, channels and transport proteins serve different functions or have different properties during distinct phases in the life cycle of a neuron. Consequently their expression patterns change during development (Schlick et al. 2010) and in some cases they undergo an isoform switch during differentiation (Vance et al. 1998). During development Ca_Vs serve in the regulation of neuronal mobility, path-finding and synapse formation (Pravettoni et al. 2000; Zheng and Poo 2007). These functions likely require different subunit isoforms or splice variants than in differentiated neurons. Moreover, neurons possess the unique ability to alter expression and composition of synaptic proteins in an activity-dependent manner. It can be expected that any of these changes also go hand in hand with altered subunit compositions or splice variant expression of Ca_Vs. At present hardly any information on the differential expression and subunit composition of Ca_Vs during neurogenesis and synaptic plasticity is available. Once the tools for analyzing expression patterns in specific populations of neurons are in place, the study of changing channel subunit combinations during differentiation and synaptic plasticity will be an important and fruitful undertaking.

2.3.1.3 Differential Targeting and Localization of Auxiliary Ca_V Subunits

Neurons are compartmentalized and structurally and functionally polarized more than any other cell type. Accordingly the composition of membrane proteins differs greatly between the input side, the somato-dendritic compartment, and the output side, the axonal compartment. Moreover, pre- and postsynaptic membranes differ from extrasynaptic membrane domains in composition of the lipid and protein content. Such compartmentalization requires complex targeting mechanism for most of the membrane proteins. However, Ca_Vs serve important functions in both the pre- and postsynaptic compartment. Whereas the functional expression and targeting of calcium channels is unique in each neuronal cell type, an overall preference exists of Ca_V2 and Ca_V1 channels for the pre- and postsynaptic compartment, respectively. The specific interaction of Ca_V2 channels with the presynaptic fusion apparatus has been shown to contribute to this differential targeting (Mochida et al. 2003; Szabo et al. 2006; Simms and Zamponi 2012). It can be expected that the auxiliary calcium channel subunits also display differential targeting properties in neurons. If they

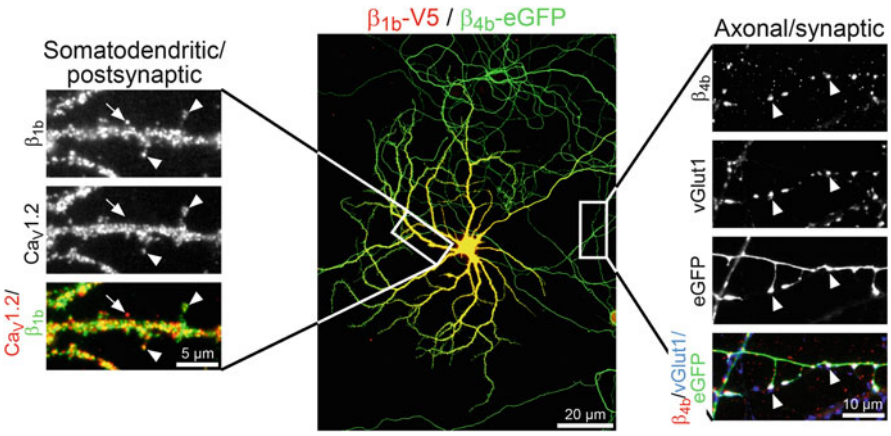

Fig. 2.2 Isoform-specific localization of V5-tagged β subunits in cultured hippocampal neurons. *Center*: A 17 DIV cultured hippocampal neuron cotransfected with a V5-tagged β_{1b} and an eGFP-tagged β_{4b} subunit. The distribution of β_{1b}-V5 is confined to the somatodendritic compartment (*yellow neurites*) whereas β_{4b}-eGFP expression is high throughout the axon and the axonal branches (*green neurites*). *Left*: V5-tagged β_{1b} co-localizes with membrane expressed HA-tagged $Ca_V1.2$ channel clusters along the dendrites and in dendritic spines (*arrowheads*). Note that some β_{1b} clusters (*arrow*) do not colocalized with $Ca_V1.2$, indicative of an association with a different Ca_V α_1 subunit. *Right*: Example of the presynaptic localization of a β subunit in a triple-labelling experiment. Fluorescence of soluble eGFP allows to morphologically identify axons with their short axonal branches with presynaptic terminals identified by staining for the vesicular glutamate transporter (vGlut1). The V5-tagged β_{4b} isoform specifically accumulates in presynaptic terminals (examples indicated by *arrowheads*). For more details see Obermair et al. (2010)

formed preferential complexes with specific α_1 subunits, their targeting mechanisms would drag the auxiliary subunits along. $\alpha_2\delta$ and β subunits would not require independent targeting mechanisms. However, if the auxiliary subunits possessed targeting mechanisms independent of α_1 subunits, they could contribute to the differential distribution of the channels in the pre- and postsynaptic compartments.

Previously we have employed the expression of epitope-tagged β subunits and subsequent immunofluorescence labeling with a single antibody as a powerful approach to analyze their targeting behavior (Obermair et al. 2010). On the one hand all four examined β subunit isoforms and two β_1 and β_2 splice variants were found in the somato-dendritic as well as in the axonal compartment. Importantly, the β_1 splice variants were less efficiently targeted to the distal axon, indicating a preferential role of in the postsynaptic/somatodendritic compartment (Fig. 2.2). However, even though the β_1 isoform was poorly targeted to the distal axon, it could be incorporated into the nerve terminal like β_2, β_3, and β_4. This observation is consistent with the great promiscuity of α_1/β interactions observed upon heterologous coexpression and indicates that in neurons the affinities of specific β-AID pairs to each other (De Waard et al. 1995) by themselves do not determine the specificity of α_1/β assemblies.

The great promiscuity in the interaction of β subunits with distinct α_1 subunits allows for the differential modulation of Ca_Vs by the association and dissociation with different β subunits (Obermair et al. 2008). Such a dynamic exchange of neuronal β isoforms with Ca_V1 channels has recently been demonstrated in differentiated skeletal myotubes (Campiglio et al. 2013). Thus, in neurons which express all β isoforms, shifts in the relative expression or local concentration of functionally distinct channel subunits may change the equilibrium between the subunit partners and thus the subunit composition of the channel complexes.

Neuronal $\alpha_2\delta$ subunits display regional differences in their expression levels in brain (Cole et al. 2005; Taylor and Garrido 2008). Nevertheless, similar to β subunits three out of four $\alpha_2\delta$ subunits are simultaneously expressed in neurons of the CNS (Schlick et al. 2010; Nimmervoll et al. submitted). $\alpha_2\delta$ subunits are abundantly expressed in the neuronal plasma membrane (Bauer et al. 2009; Müller et al. 2010) and one common feature is their localization in presynaptic terminals. Accordingly $\alpha_2\delta$-1 to -3 isoforms localize to synapses upon overexpression in hippocampal neurons and can interact with presynaptic calcium channels (Hoppa et al. 2012; Nimmervoll et al. submitted). In the hippocampus immunostaining with a monoclonal antibody revealed a preferential localization of $\alpha_2\delta$-1 in mossy fibre terminals in the CA3 region of the hippocampus (Taylor and Garrido 2008). Recently characterized $\alpha_2\delta$-3 mutants in C. elegans (*unc-36*) and Drosophila (*straightjacket*) suggest a primary role in presynaptic calcium channel trafficking (Dickman et al. 2008; Saheki and Bargmann 2009). In addition $\alpha_2\delta$ subunits are synaptically expressed in specialized nerve cells including retinal photoreceptor cells (Mercer et al. 2011), dorsal root ganglion neurons (Bauer et al. 2009) and in synapses along the hearing pathway (Pirone et al. 2009). Apart from presynaptic localizations in the cerebellum $\alpha_2\delta$-2 is concentrated in lipid rafts, suggestive of a restricted expression in microdomains, which may be important for its interaction with $Ca_V2.1$ channels (Davies et al. 2006). Nevertheless, it is currently not known whether $\alpha_2\delta$ subunits display an isoform specific targeting pattern and to which extent their localization depends on the interaction with α_1 subunits and vice versa.

2.4 Neuronal Functions Related to Auxiliary Calcium Channel Subunits

2.4.1 Channel Trafficking and Current Modulation by β Subunits

The auxiliary $\alpha_2\delta$ and β subunits are important factors for trafficking Ca_Vs to the plasma membrane and possibly for stabilizing them in functional signaling complexes. Because an increased density of functional channels in the synapse is expected to raise the efficacy of synaptic release, regulating membrane expression may act as an efficient mechanism to modulate synaptic function. Especially the

β subunit has long been known to strongly increase calcium current density upon coexpression in HEK cells or X. laevis oocytes (reviewed in Dolphin 2003; Buraei and Yang 2010). Recent studies provided insight into the role of β subunits in membrane targeting of calcium channels in native cells and tissues (Tab. 2.1; reviewed in Buraei and Yang 2010).

For example, antisense knockdown of β subunits in cultured rat dorsal root ganglion neurons strongly decreased barium currents through endogenous calcium channels (Berrow et al. 1995). We could recently show that mutation of the AID in $Ca_V1.2$ channels completely prevented the surface expression of $Ca_V1.2$ in cultured hippocampal neurons (Obermair et al. 2010). Furthermore overexpression of β subunits substantially increased surface expression of $Ca_V1.2$ channels, indicating that the abundance of β subunits may present a limiting factor for the membrane expression of Ca_Vs, and that the number of channels in the neuronal membrane can indeed be regulated by the amount of available β subunits.

The mechanism by which β subunits enhance membrane expression has long been a matter of discussion. Previously it has been suggested that β enables ER export of α_1 subunits by masking an ER retention signal within the I-II intracellular loop of Ca_V1 α_1 subunits (Bichet et al. 2000). Using an elegant combination of electrophysiology and quantification of channel surface expression in HEK cells, Fang and Colecraft have systematically characterized the contribution of all intracellular domains of $Ca_V1.2$ for the β-mediated surface expression (Fang and Colecraft 2011). These experiments clearly demonstrated that the I-II linker contains a putative ER export motif and that the β-dependent increase in surface expression may require a C-terminus-dependent rearrangement of intracellular domains, thereby overcoming retention signals within the other cytoplasmic loops. Furthermore, two recent studies demonstrated that association with a β subunit prevents the proteasomal degradation of the respective α_1 subunits, thereby stabilizing and increasing the surface expression (Altier et al. 2011; Waithe et al. 2011).

In addition to effects of β subunits on membrane targeting, β subunits are powerful modulators of the channel's gating properties. Upon coexpression in heterologous cells β subunits enhance the voltage-dependent activation and inactivation. The most notable isoform-specific effect is the strong inhibition of voltage-dependent inactivation by the palmitoylated β_{2a} (Qin et al. 1998).

There are multiple lines of evidence demonstrating that β subunits modulate calcium channel functions in neurons. For example overexpression of β_{2a} and β_{4b} in hippocampal neurons induce depression and paired-pulse-facilitation of autaptic synapses, most likely by a differential modulation of the current properties of presynaptic Ca_Vs (Xie et al. 2007). Moreover, Ca_V2 channels are subject to presynaptic inhibition by hormones and neurotransmitters through G-protein coupled receptors linked to $G_{i/o}$ via Gβγ. This inhibition, which may be involved in short-term synaptic plasticity, is voltage-dependent and depends on the presence of the β subunit in an isoform-specific manner (reviewed in Dolphin 2003). It appears that G-protein βγ association with Ca_V2 channels antagonizes the effects of the β subunit on voltage-dependent activation. The larger the hyperpolarizing effect of the

β subunit, the larger the G-protein induced inhibition. Conversely, the β subunits increase the dissociation of Gβγ and thus relieve inhibition during paired pulse facilitation (Canti et al. 2000; Feng et al. 2001).

A similarly strong β subunit dependence on GPCR modulation of Ca_Vs via Gq-proteins has been reported. Both potential mechanisms, inhibition of Ca_V channels by phosphatidylinositol 4,5-bisphosphate (PIP_2) depletion or arachidonic acid generation, are strongly abated upon coexpression of the palmitoylated β_{2a} isoform (Heneghan et al. 2009; Suh et al. 2012). Thus, lipid modulation together with the nature of the Ca_V-associated β subunit emerges as a powerful modulator of neuronal excitability or neurotransmitter release (Striessnig 2009).

The RGK (Rad, Rem, Rem2, Gem/Kir) family of small monomeric GTP-binding proteins are potent inhibitors of neuronal Ca_Vs; both when heterologously expressed and in native cells including neurons (Chen et al. 2005; reviewed in Buraei and Yang 2010). Multiple inhibitory mechanisms have been suggested including inhibition of membrane expression due to binding to and sequestration of the β subunit and current inhibition of channels preexisting in the membrane. Although calcium current inhibition by RGK proteins absolutely depends on the β subunit and its properties are reminiscent of Gβγ inhibition (see above), recent mutagenesis studies indicate that they use distinct mechanisms (Fan et al. 2010). Whether this potent inhibitory mechanism actually is in effect in synapses, and if so, how it would be activated in neurons remains to be investigated.

Ca_Vs can be regulated by phosphorylation of the α_1 subunits as well as the β subunits. PKA, PKC, CaMKII, PI3K/Akt and MAPK have all been shown to phosphorylate β subunits and modulate calcium currents in a β-dependent and isoform-specific manner (Dolphin 2003). For some of these protein kinases the phosphorylation sites in the β subunit have been identified and mutation thereof has been demonstrated to abolish the modulatory effects. If active in the synapse, any of these mechanisms might be fit to modulate synaptic transmission. Moreover, isoform-specific differences in phosphorylation add to the functional heterogeneity and potential specificity of modulatory mechanisms in synapses expressing channels of different subunit composition. However, whereas the physiological role of calcium channel phosphorylation in the fight-or-flight response is well established in the heart (Fuller et al. 2010), a similar role in presynaptic function, and particularly the involvement of β subunits is still elusive.

Ca_Vs functionally interact directly and indirectly via the β subunit with a number of other ion channels and signaling proteins including calcium-activated K^+ channels, bestrophin, the ryanodine receptor, dynamin, synaptotagmin I, and the Rab interacting protein RIM1. Most of these proteins can be found in synapses and therefore could potentially function as up- or downstream modulators of synaptic function. As of today the best candidate for such a modulation is RIM1, which is essential for synaptic transmission and plasticity and binds to β subunits with high affinity (Kiyonaka et al. 2007). This interaction appears to affect presynaptic function in two ways. First it is important for docking neurotransmitter vesicles to Ca_V2 channels, and secondly it modulates voltage-dependent inactivation of the

channel. In heterologous expression systems this interaction was observed with any of the β isoforms coexpressed with Ca_V2 channels. Whether in the context of the synapse the RIM1-β interaction displays more isoform specificity remains to be investigated.

2.4.2 *Channel Trafficking and Current Modulation by $\alpha_2\delta$ Subunits*

The roles of $\alpha_2\delta$ subunits in synaptic function are less well defined than those of the β subunits. When heterologously expressed all $\alpha_2\delta$ subunit isoforms can modulate the trafficking and/or the current properties of Ca_V α_1 subunits (reviewed in Arikkath and Campbell 2003; Davies et al. 2007; Obermair et al. 2008). In skeletal and cardiac muscle, for example, $\alpha_2\delta$-1 determines the typical current properties of the respective L-type Ca_Vs (Obermair et al. 2005, 2008; Tuluc et al. 2007; Gach et al. 2008). Therefore $\alpha_2\delta$-1 is an important determinant of action potential duration in cardiac myocytes (Tuluc et al. 2007; Templin et al. 2011). When coexpressed with neuronal P/Q- or N-type channels all three neuronal $\alpha_2\delta$ subunits cause an increase in current density (e.g., Davies et al. 2007). Conversely shRNA depletion of $\alpha_2\delta$-1 in the skeletal muscle expression system strongly reduced heterologously expressed $Ca_V2.1$ and $Ca_V2.2$ currents (Obermair et al. 2008).

Based on these results a role of $\alpha_2\delta$ subunits in triggering neurotransmitter release, which is directly related to the number of presynaptic Ca_Vs (Schweizer et al. 2012), was to be expected. Nevertheless, conflicting results have been reported on the effects of GBP administration on synaptic functions. Whereas acute application of these drugs has only mild effects (if any) on calcium currents (Alden and Garcia 2001; Kang et al. 2002; Micheva et al. 2006; Davies et al. 2007; Dooley et al. 2007) chronic application of GBP has been shown to reduce both native N-type and heterologously expressed P/Q-type calcium currents by about 50 % (Hendrich et al. 2008). Thus it is meanwhile well established that chronic GBP treatment interferes with calcium channel trafficking to the cell surface (Tran-Van-Minh and Dolphin 2010; Dolphin 2012). The importance of $\alpha_2\delta$ subunits in presynaptic functions (see Table 2.1 and Fig. 2.3) related to their role in Ca_V targeting is further supported by the upregulation of $\alpha_2\delta$ subunits in animal models of neuropathic pain (Bauer et al. 2009; Lu et al. 2010) and impaired Ca_V trafficking after chronic GBP and PG treatment (Bauer et al. 2009; Tran-Van-Minh and Dolphin 2010). Also the recently identified interaction of $\alpha_2\delta$-1 with mutant prion protein was shown to impair proper membrane trafficking of the calcium channel complex and consequently reduced glutamatergic transmission in CGNs (Senatore et al. 2012). Indeed chronic treatment with GBP significantly reduced synaptic release efficacy as measured by high KCl-induced FM-dye release in cultured hippocampal neurons (Nimmervoll et al. submitted). This GBP mediated inhibition of synaptic release was augmented in cultures from $\alpha_2\delta$-3 null neurons, indicating that $\alpha_2\delta$-3 partially compensated for the effects of GBP on $\alpha_2\delta$-1 and -2. A similarly strong effect of chronic application

Table 2.1 Effects of auxiliary calcium channel subunits on synaptic functions

	Basic modulatory effect	Mechanism (mediator)	Evidence[a]
β	Trafficking and membrane expression	Direct	Neef et al. (2009), Obermair et al. (2010), and Li et al. (2012)
		RGK GTPases	Correll et al. (2008) and Leyris et al. (2009)
		Aminopyridines	Wu et al. (2009)
	Modulation of the calcium currents	G-protein modulation	Feng et al. (2001), Dolphin (2003), and Heneghan et al. (2009)
		RIM	Kiyonaka et al. (2007), Gebhart et al. (2010), and Gandini and Felix (2012)
		RGK GTPases	Correll et al. (2008) and Leyris et al. (2009)
		PIP2	Correll et al. (2008) and Suh et al. (2012)
		Gating properties	Xie et al. (2007)
		Aminopyridines	Wu et al. (2009)
	Linking calcium channels to release sites	RIM	Kiyonaka et al. (2007) and Gandini et al. (2011)
		Synaptotagmin	Vendel et al. (2006)
$\alpha_2\delta$	Trafficking and membrane expression	Direct	Dickman et al. (2008), Hendrich et al. (2008), Ly et al. (2008), Bauer et al. (2009), Saheki and Bargmann (2009) Martinez-Hernandez et al. (2011), Hendrich et al. (2012), Hoppa et al. (2012), and Nimmervoll et al. (submitted)
		PrP interaction	Senatore et al. (2012)
	Synapse formation/structural organization	Not known	Wycisk et al. (2006a)
		Not known	Kurshan et al. (2009)
		Thrombospondin	Eroglu et al. (2009)
		Vesicular signaling	Alix et al. (2008)
	Linking calcium channels to release sites	Not known	Hoppa et al. (2012) and Nimmervoll et al. (submitted)

[a]Including some indirect evidence for synaptic function

of PG on synaptic transmission between dorsal root ganglion and dorsal horn neurons, which primarily express $\alpha_2\delta$-1, has been observed (Hendrich et al. 2012).

shRNA knockdown of $\alpha_2\delta$-1 in hippocampal neurons reduced presynaptic expression of $Ca_V2.1$ and concomitantly synaptic release probability induced by single action potentials (Hoppa et al. 2012). Conversely, $\alpha_2\delta$ subunit overexpression increased presynaptic calcium channel density and release probability. However, at the same time the presynaptic calcium signal was significantly reduced. This suggests that $\alpha_2\delta$ subunits may be involved in linking presynaptic Ca_Vs to the release site. Surprisingly and in contrast to increased release probability upon

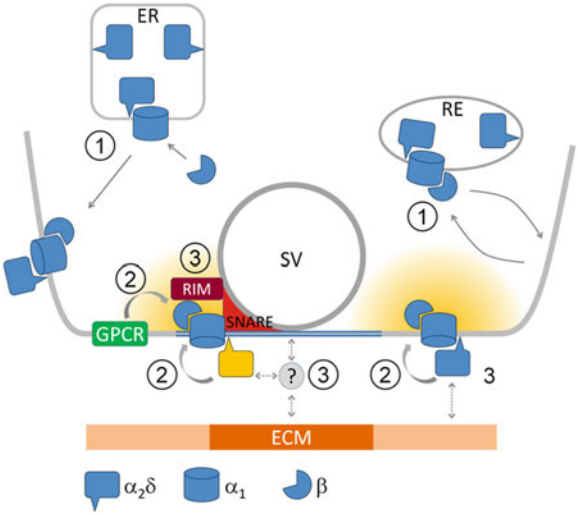

Fig. 2.3 Model summarizing the putative effects of the auxiliary $\alpha_2\delta$ and β subunits in the presynaptic compartment: (1) Trafficking: export from the endoplasmic reticulum (ER) by β; trafficking from recycling endosomes (RE) by $\alpha_2\delta$. (2) Ca_V current modulation: modulation by distinct β isoforms either directly, or by mediating modulatory mechanism in a β-isoform dependent manner (e.g. GPCR modulation); modulation by distinct $\alpha_2\delta$ subunits (indicated in *blue/orange*) by association/dissociation. (3) Linking calcium channels to the release site (*SV*, synaptic vesicle): β subunits via binding to RIM and SNARE proteins; $\alpha_2\delta$ subunits by association with a potential extracellular ligand and/or the extracellular matrix (*ECM*). Thereby individual $\alpha_2\delta$ isoforms (indicated in *orange*) may link calcium channels better to the release site by interaction with special ECM components or a specialized lipid domain in the synaptic membrane (indicated by *blue double line*)

single action potentials, in our own experiments we observed a slight reduction of synaptic FM-dye release during sustained depolarization upon $\alpha_2\delta$-1 overexpression (Nimmervoll et al. submitted). Thus, it is possible that $\alpha_2\delta$-1 overexpression one the one hand inhibits calcium influx and consequently synaptic release during prolonged depolarization like trains of action potentials or high KCl. On the other hand this might increase release probability due to a tighter association of calcium channels with the releases site upon single action potentials (Hoppa et al. 2012). Alternatively the reduction in the presynaptic calcium influx may be a consequence of a reduction of the action potential duration upon $\alpha_2\delta$ subunit overexpression (Hoppa et al. 2012). Thus, besides regulating synaptic transmission, $\alpha_2\delta$ subunits may control neuronal excitability, for example by increasing somatodendritic calcium channels and thus the coupling to calcium-activated potassium channels as previously characterized for BK channels (Berkefeld et al. 2006). Apart from regulating surface expression the specific functions of $\alpha_2\delta$ subunits on somatodendritic calcium channels have so far not been studied. While chronic treatment with GBP strongly reduced synaptic FM-dye release (see above), we found release kinetics to be unaffected in a double $\alpha_2\delta$-1 knockdown/$\alpha_2\delta$-3 knockout model. This strongly implicated the remaining

$\alpha_2\delta$-2 subunit to compensate for the loss of $\alpha_2\delta$-1 or $\alpha_2\delta$-3 dependent trafficking and modulation functions. However, it may also suggest that $\alpha_2\delta$-2 is chiefly involved in regulating transmitter release, likely by an association with presynaptic P/Q-type channels, which solely determine the kinetic properties of KCl-dependent FM-dye release in cultured hippocampal neurons (Nimmervoll et al. submitted).

A preferential correlation of $Ca_V2.1$ and $\alpha_2\delta$-2 expression was observed when quantifying overall Ca_V mRNA abundance (Schlick et al. 2010). Importantly, these recent findings on the differential synaptic roles of $\alpha_2\delta$ isoforms are further in agreement with the phenotypes of isoform specific $\alpha_2\delta$ subunit null mice. While $\alpha_2\delta$-1 and $\alpha_2\delta$-3 knockout mice display only mild overall CNS phenotypes (Fuller-Bicer et al. 2009; Neely et al. 2010), $\alpha_2\delta$-2 knockout or mutant (*ducky*, see box) mice display epilepsy and ataxia and show severely impaired cerebellar development (Brodbeck et al. 2002; Ivanov et al. 2004).

The altered release probabilities observed in hippocampal neurons from double knockout/knockdown cultures provided the first indirect evidence, that in a presynaptic bouton, which simultaneously expresses three $\alpha_2\delta$ isoforms, a single $\alpha_2\delta$ isoform may preferentially associate with a specific Ca_V α_1 subunit partner. However, it did not allow any conclusion on the nature or stability of this interaction and many studies described above favor a nonselective interaction of α_1 and $\alpha_2\delta$ subunits, similar to the promiscuity of the interaction with β subunits. In mouse chromaffin cells, for example, PG treatment blocked exocytosis by non-selectively inhibiting Ca_V1, $Ca_V2.1$ and $Ca_V2.2$ channels (Hernandez-Vivanco et al. 2012), further indicating the interaction of $\alpha_2\delta$-1 with distinct α_1 subunits. In agreement with these observations both indirect and direct evidence accumulated over the last years suggesting that $\alpha_2\delta$ subunits may not be tightly associated with channel complexes and also exist independent of the complex. In our own studies in skeletal muscle cells we could show that free $\alpha_2\delta$ exists in the plasma membrane without α_1 subunits, and that membrane expression of $\alpha_2\delta$ subunits appears to be independently regulated (Flucher et al. 1991; Obermair et al. 2005, 2008; Schredelseker et al. 2005). Similarly, not all cerebellar $Ca_V2.1$ α_1 subunits seem to be associated with an $\alpha_2\delta$-2 subunit (Davies et al. 2006). As mentioned above, proteomics of mammalian Ca_V2 channels did not identify $\alpha_2\delta$ subunits as core components of the complex (Müller et al. 2010). Thus, with the exception of cellular model systems that express an exclusive or at least preferential set of Ca_V α_1, β and $\alpha_2\delta$ subunits, little information exists on which and how $\alpha_2\delta$ subunits interact with the Ca_V complex.

2.4.3 Functions Independent of the Calcium Channel Complex

2.4.3.1 $\alpha_2\delta$ Subunits and Synapse Formation

Traditionally the auxiliary Ca_V subunits $\alpha_2\delta$ and β have been envisioned as stable components of the Ca_V complex in a 1:1 ratio with α_1 subunits. However, recently experimental evidence accumulated that suggests cellular function of these two

proteins that are in part or entirely independent of the Ca_V complex. As for the $\alpha_2\delta$ subunits several studies point towards a major calcium channel independent contribution to synapse formation, likely by interaction with components of the extracellular matrix (Fig. 2.3). $\alpha_2\delta$-1 has been shown to act as a receptor for thrombospondin, an astrocyte-secreted protein that promotes CNS snaptogenesis (Eroglu et al. 2009). Overexpression of $\alpha_2\delta$-1 strongly promoted, and shRNA knockdown inhibited excitatory synapse formation in cultured retinal ganglion cells. GBP treatment also inhibited synapse formation and the mechanism was shown to involve the $\alpha_2\delta$-1 VWF domain. Using a forward genetic screen, Drosophila mutants for the $\alpha_2\delta$-3 (*straightjackt; stj*) isoform have been identified which show defects in presynaptic Ca_V localization and synaptic function (Dickman et al. 2008). By further analyzing the phenotypes of the $\alpha_2\delta$-3 (*stj*) null mutants, it became evident that motoneurons failed to develop normal synapses (Kurshan et al. 2009). Interestingly, this phenotype was independent of the Drosophila pore forming α_1 subunit (*cacophony*) since *cacophony* null mutants showed no defect in synapse formation. Mutant (*du, du^{2J}, entla*) and targeted knockout mice for $\alpha_2\delta$-2 display altered morphology and reduced calcium currents in Purkinje cells (Barclay et al. 2001; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006), also suggesting a defect in synapse formation. Finally, it has been shown that the spontaneous mouse mutant of $\alpha_2\delta$-4 (Cacna2d4) causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a).

All these strong effects of loss-of-$\alpha_2\delta$-function on synapse structure and formation where revealed in model systems that primarily express only one $\alpha_2\delta$ isoform, such as retinal ganglion cells, Drosophila motoneurons, and mammalian photoreceptors. In cellular systems which express more than one $\alpha_2\delta$ isoform, such as CNS neurons, both calcium channel dependent and independent functions of $\alpha_2\delta$ subunits appear to be subject to compensation by other $\alpha_2\delta$ isoforms. Recently we analyzed the density of functional synapses of $\alpha_2\delta$ loss-of-function models (Nimmervoll et al. submitted). We found that synapse formation was still close to normal in $\alpha_2\delta$-3 deficient cultured hippocampal neurons in which $\alpha_2\delta$-1 was knocked down or $\alpha_2\delta$-1 and $\alpha_2\delta$-2 were chronically blocked with GBP. Thus, it seems that the contribution of individual $\alpha_2\delta$ isoforms to synapse formation is limited in neurons expressing three different $\alpha_2\delta$ isoforms. This is further supported by the recent characterization of $\alpha_2\delta$-3 knockout mice, which did not reveal overall effects on synapse formation (Neely et al. 2010). To answer this question, it will ultimately be necessary to study synapse formation in CNS neurons lacking all $\alpha_2\delta$ isoforms.

2.4.3.2 β Subunits and Transcriptional Regulation

The first indication of calcium channel independent functions of β subunits came from isolated observations of heterologously expressed β subunits localized in the cell nuclei (Colecraft et al. 2002; Hibino et al. 2003; Beguin et al. 2006).

Interestingly, the truncated chicken β_{4c} isoform associated with heterochromatin protein 1 (HP1) a nuclear protein involved in gene silencing (Hibino et al. 2003). In 2009 we localized the endogenous β_4 isoform in the nuclei of cerebellar granule and Purkinje cells and demonstrated in a skeletal muscle expression system that nuclear targeting of heterologous β subunits is isoform and splice variant specific ($\beta_{4b}>>\beta_{4a}=\beta_3>\beta_{1a}=\beta_{1b}=\beta_{2a}=\beta_{2b}$) and negatively regulated by electrical activity and calcium influx into nerve and muscle cells (Subramanyam et al. 2009). The finding that in immature and quiescent cells β_{4b} accumulated in the nucleus and upon the onset of electrical activity it was released from the nuclei suggested a possible role in activity dependent gene regulation. Very recently Tadmouri et al. reported that β_{4b} associates with the regulatory subunits of protein phosphatases 2A, translocates into the nucleus in an activity dependent manner, where it associates with the tyrosine hydroxilase promoter and histone H3 in complex with HP1 (Tadmouri et al. 2012). Importantly, a truncated β_4 mutant associated with juvenile myoclonic epilepsy failed to complex with B56δ and consequently did not translocate into the nucleus. These findings suggest that the neurological disease phenotype in humans and that of the β_4 knockout mouse are at least in part related to the nuclear function of the β_{4b} subunit, whereas its calcium channel dependent functions may be compensated by other β isoforms. Also β_3 subunits may function in transcriptional regulation. Recently the specific interaction of β_3 with a novel Pax6(S) transcriptional regulator has been described (Zhang et al. 2010). Upon coexpression in Xenopus oocytes β_3 is translocated into the nucleus and suppresses the transcriptional activity of Pax6(S). As Pax6 transcriptional regulators are important during development, a role of this calcium channel independent activity of β in developmental regulation has been suggested. Consistent with function in early development, morpholino knockdown of β_4 in zebrafish embryos blocked epiboly, a reorganization of cells during gastrulation (Ebert et al. 2008). Importantly, this effect could be rescued by coexpression of a β_{4a} isoform with mutated AID binding pocket, again indicative of a calcium channel independent mechanism. So far no direct link of any of these nuclear functions of β subunits to synaptic function has been established. However, because these novel pathways for transcriptional regulation are activity dependent and affect developmental processes, a mechanism by which β subunit signaling provides a feedback loop from overall synaptic activity to synapse efficacy analogous to homeostatic plasticity can be envisioned.

2.4.4 Auxiliary β and $\alpha_2\delta$ Subunits and Neuronal Disease

There is little evidence for an involvement of calcium channel β subunits in neurological disease. Also, with the exception of *lethargic* (β_4-null) mice, mouse mutant and knockout models of β subunits show little to no neurological defects (see box). Loss of function phenotypes can be observed in cell types predominantly

expressing a single β isoform like skeletal (β_1) and cardiac muscle (β_2) or the retina and inner hair cells (β_2). In other cells, including most neurons, expression of other β isoforms seems to compensate the loss of the respective isoform. The β_4 subunit is the notable exception. Mutations resulting in a truncated protein have been linked to juvenile myoclonic epilepsy (Escayg et al. 2000) and the *lethargic* β_4-null mutant mouse develops severe ataxia and epileptic seizures (Burgess et al. 1997). The similarity of this phenotype to that of $Ca_V2.1$ (*tottering, leaner*) (Doyle et al. 1997) and $\alpha_2\delta$-2 (*ducky*) (Barclay et al. 2001) mutants and their predominant expression in cerebellum (see above) indicates that in some cerebellar neurons this set of subunits forms an essential channel complex. Loss of any one of its components cannot be fully compensated by other isoforms. Alternatively, the neurological β_4 phenotype could arise from an exclusive nuclear function of this subunit in gene regulation.

As mentioned above, $\alpha_2\delta$-dependent functions can be exerted as calcium channel subunits on the one hand, and independent of the Ca_V complex on the other. For example meanwhile it is well established that $\alpha_2\delta$-1 is strongly upregulated in dorsal root ganglion neurons in animal models of neuropathic pain (Luo et al. 2001). The beneficial effect of GBP and PG in neuropathic pain (Field et al. 2006) most likely results from impairing excess $\alpha_2\delta$ subunit trafficking (Bauer et al. 2009). As a possible mechanism inhibiting recycling of $\alpha_2\delta$ subunits from the endosomes has been described (Tran-Van-Minh and Dolphin 2010). *straightjacket* mutants also display altered heat nociception and CACNA2D3 ($\alpha_2\delta$-3) single nucleotide polymorphisms (SNPs) in humans have been linked to central pain processing (Neely et al. 2010). This phenotype, which is likely caused by a change in local CNS excitability, could both be explained by a defect in Ca_V trafficking and synapse formation. Mutant (*du, du^{2J}, entla*) and knockout mice for $\alpha_2\delta$-2 display altered morphology and reduced calcium currents in Purkinje cells as well as cerebellar ataxia and absence epilepsy (Barclay et al. 2001; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006). In humans the $\alpha_2\delta$-2 gene (CACNA2D2) has been discussed as a potential tumor suppressor gene (Hesson et al. 2007) and in the context of childhood absence epilepsy (Chioza et al. 2009). Indeed, very recently Edvardson et al. identified the first human mutation in the CACNA2D2 gene associated with an early infantile epileptic encephalopathy (Edvardson et al. 2013). A spontaneous mouse mutant of $\alpha_2\delta$-4 (Cacna2d4) causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a) and a human CACNA2D4 mutation underlies a slowly progressing cone dystrophy associated with night blindness (Wycisk et al. 2006b). Finally, clinical applications of GBP and PG provide an important link between $\alpha_2\delta$ subunits and neuronal disease. Besides their effectiveness in neuropathic pain conditions, which is most likely mediated by binding to $\alpha_2\delta$-1 (Field et al. 2006), both drugs have proven efficacy in epilepsy and generalized anxiety disorders (Bryans and Wustrow 1999; Johannessen Landmark 2008). The recently identified interaction of mutant prion protein with $\alpha_2\delta$-1 may provide an essential disease mechanism in the pathophysiology of prion diseases, namely by disrupting cerebellar glutamatergic neurotransmission (Senatore et al. 2012).

Box: Insight into Neuronal Functions of Auxiliary Subunits from Knockout and Mutant Animal Models

- **β_1 (Cacnb1):** Mice with a disruption of the β_1 isoform die after birth due to respiratory failure (Gregg et al. 1996). Similarly the paralyzed zebrafish mutant *relaxed* is a functional β_1 null mutant (Schredelseker et al. 2005) displaying disturbed skeletal muscle function. Rescue of the skeletal muscle phenotype in β_1 knockout mice by expression of the murine β_{1a} cDNA under the control of the human skeletal muscle actin promoter did not show any obvious neuronal phenotype, suggesting that loss of β_1 can be compensated by other β subunits (Ball et al. 2002). Nevertheless, detailed analysis of neuronal phenotypes is still pending.
- **β_2 (Cacnb2):** Mice with a targeted deletion of β_2 die during embryonic development due to cardiac failure (Ball et al. 2002; Weissgerber et al. 2006). Mice in which the lethal phenotype was rescued by the expression of β_2 under a cardiac promoter did not display an obvious CNS phenotype. Nevertheless, these mice displayed altered retinal morphology highlighting the importance of β_2 in membrane expression of $Ca_V1.4$ channels, which are exclusively expressed in the retina. Furthermore these mice are deaf due to reduced $Ca_V1.3$ membrane expression in inner hair cells, revealing their importance in the L-type channel dependent synapse (Neef et al. 2009).
- **β_3 (Cacnb3):** Deletion of the β_3 isoform resulted in reduced N-type currents in the hippocampus, suggesting a preferential interaction of β_3 with $Ca_V2.2$, as well as enhanced LTP and hippocampus-dependent learning. Lower anxiety, increased aggression and nighttime activity further indicate a general imbalance in neuronal calcium handling (Namkung et al. 1998; Murakami et al. 2007; Jeon et al. 2008).
- **β_4 (Cacnb4):** The spontaneous β_4 mouse mutant *lethargic* displays ataxia and epileptic seizures (Burgess et al. 1997) and the major phenotype arises from disrupted P/Q-type signaling in the cerebellum (similar to $Ca_V2.1$ *tottering* and $\alpha_2\delta$-2 *ducky* mutant animals), highlighting the preferential local association of β_4 with $Ca_V2.1$ and $\alpha_2\delta$-2. β_4 mutants display the strongest CNS phenotype of all β knockout mice, suggesting limited potential for compensation by other isoforms. It is further tempting to speculate that this may at least in part relate to the unique property of β_4 subunits in the regulation of gene transcription.
- **$\alpha_2\delta$-1 (Cacna2d1):** As previously inferred from a modeling study (Tuluc et al. 2007), $\alpha_2\delta$-1 knockout mice display reduced cardiac L-type currents (Fuller-Bicer et al. 2009). However, the mice do not show any obvious neuronal phenotype.
- **$\alpha_2\delta$-2 (Cacna2d2):** The naturally occurring $\alpha_2\delta$-2 mutants (*du, du^{2J}, entla*) as well as targeted knockout mice display epilepsy and ataxia

(continued)

(continued)
and show severely impaired cerebellar development (Barclay et al. 2001; Brodbeck et al. 2002; Brill et al. 2004; Ivanov et al. 2004; Donato et al. 2006). Together with the β_4 mutant *lethargic* and the $Ca_V2.1$ mutant *tottering* this mirrors the association of these subunits in the cerebellum. Due to the severe cerebellar phenotype, other CNS functions (e.g., in the hippocampus) have until today not been analyzed.

- **$\alpha_2\delta$-3 (Cacna2d3):** Mice with a targeted deletion of $\alpha_2\delta$-3 display defects in pain processing (Neely et al. 2010), the precise mechanism for this effect has not yet been elucidated. Synapse formation in hippocampal neurons appears to be normal (Nimmervoll et al. submitted). However, these mice have impaired hearing and a reduced auditory startle response, which is likely caused by a defect in synapse formations along the auditory pathway (Pirone et al. 2009). The Drosophila $\alpha_2\delta$-3 mutant *straightjacket* displays defects in presynaptic channel trafficking, motoneuron synapse formation, and altered heat nociception (Dickman et al. 2008; Kurshan et al. 2009; Neely et al. 2010). A mutant of the C. elegans $\alpha_2\delta$ subunit *unc-36* shows impaired synaptic function likely due to impeded presynaptic channel trafficking (Saheki and Bargmann 2009).
- **$\alpha_2\delta$-4 (Cacna2d4):** A spontaneous mouse mutant of $\alpha_2\delta$-4 causes structural and functional abnormalities of retinal ribbon synapses associated with the loss of rods (Wycisk et al. 2006a). The human CACNA2D4 mutation underlies a slowly progressing cone dystrophy associated with night blindness (Wycisk et al. 2006b).

2.5 Conclusion

Moving the focus of calcium channel research from heterologous expression systems to differentiated cells including neurons and to the study of animal models have greatly advanced our understanding of the physiology of auxiliary Ca_V subunits. However, many of the new functional insights have also revealed our limited ability to associate their specific functions to particular molecular entities. This deficit has been further exacerbated by the growing molecular diversity of calcium channel subunits brought about by posttranscriptional modifications like splicing and RNA editing. Therefore, future research first and foremost needs to uncover how specific Ca_V complexes are established in neurons expressing many different isoforms. As outlined above, this will require the detailed study of their expression patterns, their targeting mechanisms and their protein-protein interactions. To uncover these aspects in the context of calcium channel signalling complexes like the presynaptic compartment high- and superresolution microscopy approaches will be necessary. Finally, the static picture of molecular complexes needs to be replaced by one

of highly dynamic signalosomes, in which all the mechanisms mentioned above contribute to an equilibrium of multiple protein-protein interactions that ultimately determines the functional properties of the signalling complex. In the synapse such dynamic calcium channel complexes are critical for the activity-dependent regulation of synaptic strength and ultimately for the ability of our nervous system to learn and store new information.

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Chapter 3
Reciprocal Regulation of Neuronal Calcium Channels by Synaptic Proteins

Norbert Weiss and Gerald W. Zamponi

Abstract Voltage-gated Ca^{2+} channels represent one of the main pathways for Ca^{2+} entry into nerve terminals where they play a critical role in the control of synaptic exocytosis. It is traditionally believed that the vesicle-docking/release machinery must be located in the vicinity of the calcium source in order to trigger fast, efficient and spatially delimited neurotransmitter release. This tight coupling is mostly achieved by a physical interaction of the presynaptic calcium channel with several actors of the synaptic vesicle release machinery. Conversely, the binding of synaptic proteins regulates calcium channel activity, providing for fine control of presynaptic Ca^{2+} entry. Here, we review the current state of knowledge of the molecular mechanisms by which synaptic proteins regulates presynaptic Ca^{2+} channel activity.

Keywords SNARE protein • Syntaxin • SNAP-25 • Synaptotagmin • Munc18 • Rim • Cysteine string proteins • Huntingtin

3.1 Introduction

Voltage-gated Ca^{2+} channels (VGCCs) are plasma membrane proteins that convert an electrical signal into intracellular Ca^{2+} elevations. To date, ten genes encoding the pore-forming subunits of mammalian VGCCs have been identified. Seven genes encode the high-voltage activated (HVA) channel subfamily (comprising L-type ($Ca_V1.1$ to $Ca_V1.4$), P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$) and R-type ($Ca_V2.3$) channels) and three genes encode the low-voltage-activated (LVA) channel subfamily (composed exclusively of T-type ($Ca_V3.1$ to $Ca_V3.3$) (Ertel et al. 2000). In addition

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to the Ca_V pore-forming subunit, HVA channels are contain auxiliary subunits: β (β$_1$ to β$_4$, a 55 KDa cytosolic protein of the MAGUK (**m**embrane-**a**ssociated **gu**anylate **k**inase) family), α$_2$δ (α$_2$δ$_1$ to α$_2$δ$_4$, a 170 KDa highly glycosylated extracellular protein with a single transmembrane domain), and in some cases γ (γ$_1$ to γ$_8$, a 33 KDa transmembrane protein) (Takahashi et al. 1987). Among this wide diversity of native channels, $Ca_V2.1$ and $Ca_V2.2$ channels have been identified as the predominant Ca^{2+} channels involved in depolarization-evoked neurotransmitter release (Westenbroek et al. 1992, 1995, 1998; Olivera et al. 1994; Wheeler et al. 1994; Dunlap et al. 1995; Day et al. 1996; Timmermann et al. 2002). They support a transient Ca^{2+} microdomain of high concentration (10–50 μM) (Schneggenburger and Neher 2005) within the active zone of the synapse that is essential for the fusion of presynaptic vesicles with the plasma membrane (Llinas et al. 1992; Edwards 2007; Neher and Sakaba 2008; Weber et al. 2010). $Ca_V2.1$ channels support voltage-dependent exocytosis in the central nervous system, whereas $Ca_V2.2$ channels are critically involved at the peripheral synapses. In particular synapses, $Ca_V2.3$ channels are also expressed at sufficiently high density (Day et al. 1996; Hanson and Smith 2002) to support Ca^{2+} entry into presysnaptic terminals (Breustedt et al. 2003; Dietrich et al. 2003) and contribute to some extent to the release of neurotransmitters (Wu et al. 1998; Gasparini et al. 2001; Kamp et al. 2005). Finally, although HVA channels support voltage-dependent exocytosis, release of neurotransmitters at rest (i.e. around the resting membrane potential of neurons) has been demonstrated in some neurons and relies on LVA $Ca_V3.2$ channels (Ivanov and Calabrese 2000; Pan et al. 2001; Egger et al. 2003; Carabelli et al. 2007; Weiss and Zamponi 2012)

In order to efficiently receive Ca^{2+} signals, the vesicle-docking/ release machinery must be located in the vicinity of the source of Ca^{2+}. This is particularly important considering the high Ca^{2+} buffering capability of neurons (Foehring et al. 2009) and the consequent limited diffusion of free Ca^{2+}. In mammalian synapses, this close localization relies on the direct interaction of the Ca^{2+} channels with several members of the vesicle release machinery which is essential for fast (within 200 μs after the arrival of the action potential) and spatially delimited neurotransmitter release (Sabatini and Regehr 1996; Wadel et al. 2007). Conversely, binding of synaptic proteins potently regulates channel activity, providing a reciprocal control of Ca^{2+} entry to fine tune synaptic strength. Interested readers may also refer to the work of Atlas et al., for a discussion of other possible role of biochemical coupling of VGCCs with synaptic proteins (Atlas et al. 2001; Lerner et al. 2006; Marom et al. 2007; Hagalili et al. 2008; Atlas 2010; Cohen-Kutner et al. 2010; Marom et al. 2010; Weiss 2010).

3.2 Basic Principles of Molecular Coupling Between Voltage-Gated Ca^{2+} Channels and SNARE Proteins

SNARE proteins (**s**oluble **N**SF (*N*-ethylmaleimide-sensitive fusion protein) **a**ttachment protein **re**ceptor) comprising the Q-SNAREs syntaxin-1A/1B, SNAP-25 (synaptosomal-associated protein of 25 kDa) and R-SNARE synaptobrevin

(VAMP) (Fasshauer et al. 1998; Sutton et al. 1998) form the SNARE core complex that brings the vesicle and target membranes into close opposition, leading to fusion and exocytosis (Hanson et al. 1997; Otto et al. 1997). Not surprisingly, $Ca_V2.1$ and $Ca_V2.2$ channels are presynaptically colocalized with syntaxin-1A at nerve terminals (Cohen et al. 1991; Westenbroek et al. 1992, 1995) and have been biochemically isolated in complex with SNARE proteins (Bennett et al. 1992; Yoshida et al. 1992; Leveque et al. 1994). Molecular characterization of Ca^{2+} channels/SNARE interaction has identified a *synprint* (*synaptic protein interaction*) locus in $Ca_V2.1$ and $Ca_V2.2$ located within the intracellular loop between domains II and III of the channels (Sheng et al. 1994; Rettig et al. 1996). This motif binds syntaxin-1A and SNAP-25 (but not synaptobrevin). Further biochemical mapping of the *synprint* site has identified two distinct microdomains separated by a flexible linker that independently binds syntaxin-1A and SNAP-25 (Rettig et al. 1996; Yokoyama et al. 2005). The functional relevance of the interaction has been shown by disruption of the Ca^{2+} channel/SNAREs coupling using peptides derived from the *synprint* domain (or by direct deletion of the *synprint* site) that alters synaptic transmission (Mochida et al. 1996; Rettig et al. 1997; Harkins et al. 2004; Keith et al. 2007). However, although the *synprint* site is unambiguously of key importance for fast and efficient neurotransmitter release, there is evidence that some other channel isoforms such as T-type channels, although devoid of the consensus *synprint* site, functionally contribute to presynaptic Ca^{2+} elevations and neurotransmitter release, suggesting the existence of other molecular coupling determinants. Indeed, we recently demonstrated that syntaxin-1A and SNAP-25 biochemically interact with $Ca_V3.2$ T-type channels within the carboxy-terminal domain of the channel (Weiss et al. 2012; Fig. 3.1).

3.3 Functional Interaction of Voltage-Gated Ca^{2+} Channels with SNARE Proteins

SNARE proteins not only bring presynaptic vesicles close to the Ca^{2+} source but also potently modulate channel gating to fine tune presynaptic Ca^{2+} entries and synaptic transmission (Fig. 3.2).

3.3.1 Syntaxin-1A

The notion that SNARE proteins modulate Ca^{2+} influx through VGCC arose from electrophysiological recordings in heterologous expression systems showing that coexpression of syntaxin-1A potently modulates $Ca_V2.1$ and $Ca_V2.2$ gating by shifting the voltage-dependence of inactivation toward more negative membrane potentials (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999; Degtiar et al. 2000), thus silencing the channels and reducing presynaptic Ca^{2+} entry influx. This regulation was later confirmed in chick ciliary ganglion neurons and

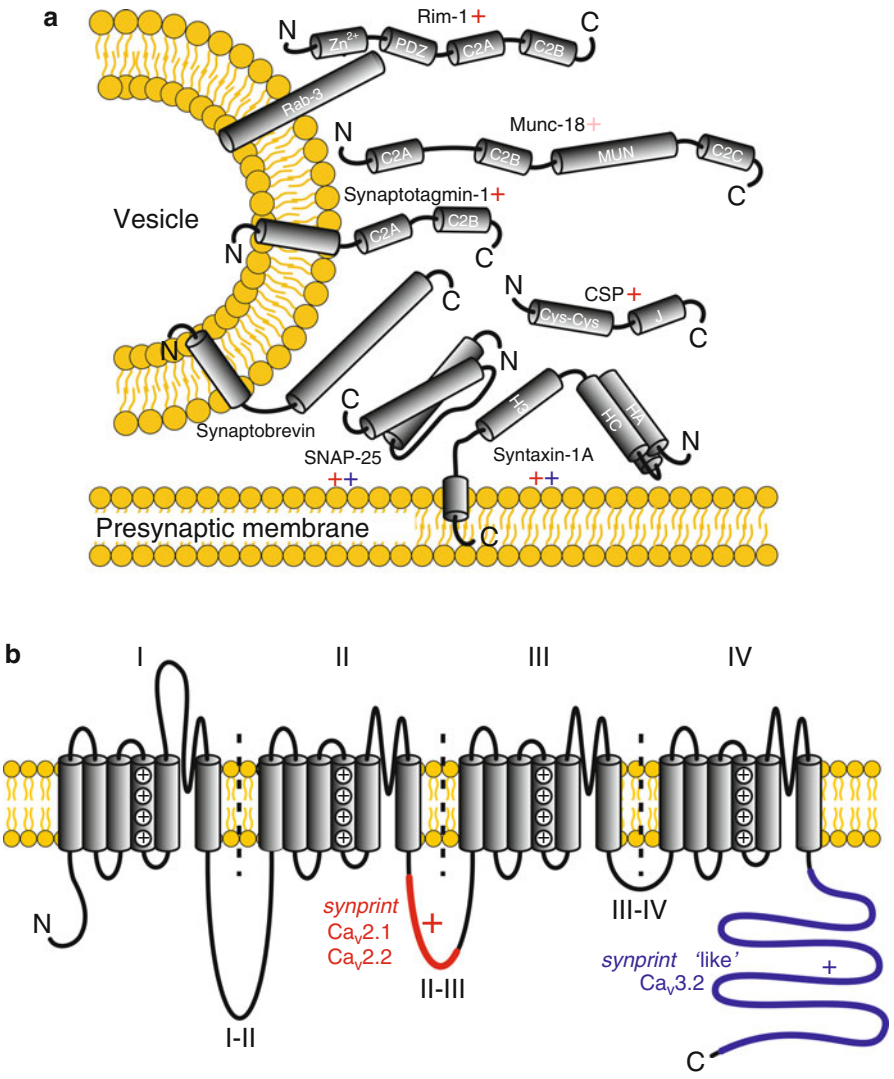

Fig. 3.1 Biochemical interactions between presynaptic Ca^{2+} channels and synaptic proteins. (**a**) Schematic representation of key synaptic proteins involved in the regulation of presynaptic Ca^{2+} channels. (**b**) Putative membrane topology of voltage-gated Ca^{2+} channels. The *synprint* domain found in $Ca_V2.1$ and $Ca_V2.2$ channels (located within the intracellular loop between domains II and III of the channel) is shown in *red*. In contrast, the *synprint* "like" domain found in $Ca_V3.2$ T-type located within the carboxy-terminal of the channel is shown in *blue*. *The red plus* "+" signs indicate proteins that interact directly with the *synprint* site, whereas the *blue plus signs* indicate those interacting with the *synprint* "like" domain of $Ca_V3.2$ T-type channels. Binding of Munc-18 with $Ca_V2.2$ channels has been shown, but the involvement of the *synprint* domain requires further investigations (Adapted from (Davies and Zamponi 2008; Abbreviations: *Rim-1* Rab-3 interacting molecule-1, *CSP* Cysteine String Proteins)

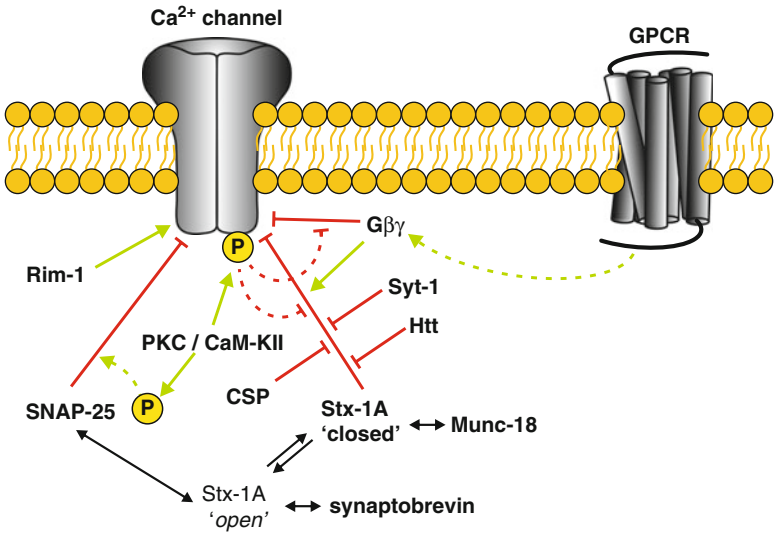

Fig. 3.2 Functional interactions between presynaptic Ca^{2+} channels, synaptic proteins and second messengers. The SNARE syntaxin-1A and SNAP-25, as well as release of free G-protein βγ dimer upon GPCR activation inhibit channel activity. In contrast, synaptotagmin-1, CSP and huntingtin prevent syntaxin-1A-dependent inhibition of the channel. Similarly, channel phosphorylation by PKC or CaM-KII prevents syntaxin-1A and Gβγ-dependent inhibition, while phosphorylation of SNAP-25 promotes its inhibitory effect. Rim-1 directly potentiates Ca^{2+} influx (Abbreviations: *Stx1A* syntaxin-1A, *Syt-1* synaptotagmin-1, *Rim-1* Rab-3 interacting molecule-1, *CSP* Cysteine String Proteins, *Htt* huntingtin, *PKC* protein kinase C, *CaM-KII* Ca^{2+}/calmodulin-dependent protein kinase II, *GPCR* G-protein coupled receptor. *Arrows in red* indicate an inhibitory regulation whereas *arrows in green* indicate a potentiation)

isolated mammalian nerve terminals (synaptosomes) upon application of botulinium neurotoxin C1 (BoNT/C1 which cleaves syntaxin-1A from its membrane anchoring domain). BoNT/C1 treatment shifted the voltage-dependence of inactivation of the channel toward depolarized potentials (Bergsman and Tsien 2000; Stanley 2003). Structure/function studies have identified the transmembrane domain of syntaxin-1A, in particularly the two cysteines (C271 and C272) (Trus et al. 2001), as well as a short stretch within the H3 helical cytoplasmic domain, as fundamental for channel modulation without a direct implication in biochemical interaction with the *synprint* domain (Bezprozvanny et al. 2000; Jarvis et al. 2002). More recently, it was also shown that besides binding the *synprint* site of $Ca_V2.2$ channels, the ten amino-terminal residues of syntaxin-1A might support inhibition of the channel (Davies et al. 2011). Although the exact molecular mechanism of syntaxin-1A mediated regulation of Ca^{2+} channels remains unclear, these results highlight the existence of two kinds of interaction of syntaxin-1A with the channel: (i) a biochemical interaction via the *synprint* domain and (ii) a functional interaction most likely involving additional yet unidentified channel determinants. Consistent with this idea, T-type Ca^{2+} channels that biochemically couple to syntaxin-1A

via different channel binding determinants than the consensus *synprint* domain, are subject to similar syntaxin-1A-dependent modulation as $Ca_V2.1$ and $Ca_V2.2$ channels. Similarly, for $Ca_V2.3$ channels, despite being devoid of the consensus *synprint* domain, various studies indicate that a similar syntaxin-1A-dependent regulation must occur (Bergsman and Tsien 2000; Wiser et al. 2002; Cohen and Atlas 2004). These observations suggest that gating modulation of VGCCs by syntaxin-1A likely involves modulatory channel determinants distinct from the anchoring domains. Dynamic intramolecular interactions between the intracellular loops of $Ca_V2.1$ and $Ca_V2.2$ channels have be reported and appear to be involved in channel gating behavior (Restituito et al. 2000; Raghib et al. 2001; Geib et al. 2002; Page et al. 2004, 2010; Sandoz et al. 2004; Agler et al. 2005; Bucci et al. 2011). Hence, channel remodeling upon syntaxin-1A binding might represent a possible consensus molecular mechanism by which syntaxin-1A modulates $Ca_V2.x$ and $Ca_V3.2$ channels in a similar manner despite distinct coupling molecular determinants. Interestingly, a mutation (A454T) that segregates with familial hemiplegic migraine patients located within the intracellular linker between domains I and II of $Ca_V2.1$ channel alters both syntaxin-1A-dependent channel gating modulation and exocytosis (Serra et al. 2010), suggesting that the I-II channel loop could play an important role in mediating syntaxin-1A modulation. In addition, a conformational switch of syntaxin-1A has been reported, that depends of the molecular partners engaged in the macromolecular complex. Hence, syntaxin-1A presents a *closed* conformation in complex with munc18 (or in isolation) and switches to an *open* conformation when in complex with SNAP-25 or synaptobrevin-2 (Dulubova et al. 1999; Brunger 2001). Interestingly, whereas syntaxin-1A in its *closed* state potently modulates $Ca_V2.2$ and $Ca_V3.2$ channel activity, coexpression of an *open* syntaxin-1A (locked open by two point mutations (Dulubova et al. 1999)) no longer alters channel gating (Jarvis et al. 2002; Weiss et al. 2012). This conformational switch of syntaxin-1A appears to be of key physiological importance since it occurs during the vesicle release cycle (Dulubova et al. 1999; Fiebig et al. 1999; Richmond et al. 2001), suggesting that syntaxin-1A may dynamically and temporally controls presynaptic Ca^{2+} entry during the exocytosis process.

3.3.2 SNAP-25

Like syntaxin-1A, SNAP-25 non-competitively binds onto the *synprint* domain of $Ca_V2.1$ and $Ca_V2.2$ channels, as well as onto the carboxy-terminal domain of $Ca_V3.2$ channels, to produce a similar inhibitory channel gating modulation (Wiser et al. 1996; Zhong et al. 1999; Weiss et al. 2012). The functional modulation produced by SNAP-25 was recently indirectly confirmed in native systems where siRNA silencing of SNAP-25 in glutamatergic neurons produced an increase of Ca^{2+} currents carried by $Ca_V2.1$ channels due to a depolarizing shift of the voltage-dependence of inactivation (Condliffe et al. 2010; Condliffe and Matteoli 2011). Interestingly, this inhibitory regulation is no longer observed when SNAP-25 is

co-expressed with syntaxin-1A (Wiser et al. 1996; Zhong et al. 1999; Jarvis and Zamponi 2001a; Weiss et al. 2012), suggesting that association of SNAP-25 with syntaxin-1A during the vesicle release cycle relieves channel inhibition, allowing timely presynaptic Ca^{2+} elevation required for membrane fusion and exocytosis (Sudhof 2004). Consistent with a key physiological importance of SNAP-25, structural or expression alterations of the protein caused by genetic mutations have been associated with numerous neuropsychiatric and neurological disorders, likely because of mis-regulation of presynaptic Ca^{2+} channels (Corradini et al. 2009). Finally, it was reported that phosphorylation of SNAP-25 by protein kinase C is required for SNAP-25-dependent inhibition of VGCCs (Pozzi et al. 2008), suggesting that like syntaxin-1A, SNAP-25-dependent modulation of channel activity may involve molecular determinants other than the *synprint* site.

3.4 Modulation of Presynaptic Calcium Channels by Non SNARE Proteins

3.4.1 Synaptotagmin-1

Although part of the vesicular release complex, synaptotagmin-1 is not as essential as syntaxin-1A or SNAP-25 in the membrane fusion process per se (Tucker and Chapman 2002), but rather works as a Ca^{2+} sensor, forming the link between presynaptic Ca^{2+} elevation and vesicular fusion, essential for fast and synchronous neurotransmission release (DeBello et al. 1993; Geppert et al. 1994; Augustine 2001; Fernandez-Chacon et al. 2001; Nishiki and Augustine 2001; Tucker and Chapman 2002; Koh and Bellen 2003; Xu et al. 2007). Indeed, synaptotagmin-1 is characterized by an amino-terminal transmembrane region anchored in the vesicle, a variable linker, and two carboxy-terminal rich negatively charged domains (C2A and C2B), each capable of binding Ca^{2+}. Hence, Ca^{2+} binding onto the C2A domain contributes to the insertion of synaptotagmin-1 into the plasma membrane, bringing vesicles docked to the plasma membrane upon Ca^{2+} elevation (Fernandez-Chacon et al. 2001). In contrast, the C2B domain has been reported to biochemically interact with the *synprint* site of $Ca_V2.1$ and $Ca_V2.2$ channels (Sheng et al. 1997). Although binding of synaptotagmin-1 has no major effect on channel gating, it reduces syntaxin-1A-dependent inhibition of $Ca_V2.2$ channels, possibly by Ca^{2+}-dependent binding competition with syntaxin-1A (Sheng et al. 1996). Hence, syntaxin-1A preferentially interacts with the channel at rest (i.e. at low Ca^{2+} level) thus preventing channel activity, whereas presynaptic Ca^{2+} elevation favors its interaction with synaptotagmin-1 and Ca^{2+} entry through VGCCs that is required for the final fusion process of docked vesicles. Moreover, a Ca^{2+}-dependent synaptotagmin-1 interaction with the $Ca_V\beta_{4a}$ auxiliary-subunit of VGCCs has been reported (Vendel et al. 2006), providing another dynamic Ca^{2+} channel/vesicle interaction (Weiss 2006).

3.4.2 Mnc-18

As for synaptotagmin-1, Munc18 belongs to the C2-domain containing protein family, and plays a fundamental role in the assembly/disassembly of the exocytosis machinery (Gulyas-Kovacs et al. 2007; Toonen and Verhage 2007). Genetic ablation of Munc-18 in mice leads to a complete loss of synaptic transmission (Verhage et al. 2000). Although a biochemical interaction of Munc-18 with $Ca_V2.2$ channel within the intracellular linker between domains II and III has been reported (Chan et al. 2007), its coexpression has no effect on channel gating (Gladycheva et al. 2004). Hence, Munc-18 appears to not be a direct modulator of Ca^{2+} channel activity but rather interferes with syntaxin-1A-dependent channel inhibition during the vesicle release cycle as previously mentioned (Dulubova et al. 1999; Brunger 2001). This occurs by stabilizing syntaxin-1A in a *closed* conformation (Jarvis et al. 2002), inhibiting Ca^{2+} channel activity and non-necessary presynaptic Ca^{2+} entry in the absence of docked vesicle.

3.4.3 Rim-1

Rim (Rab-3 interacting molecule) is also part of a family of vesicle-associated proteins whose members share C2 domains. By interacting with numerous components of the presynaptic active zone such as SNAP-25 or synaptotagmin-1 (Coppola et al. 2001), it forms a protein scaffold by participating in the docking and fusion of presynaptic vesicles (Wang et al. 2000; Betz et al. 2001; Coppola et al. 2001; Ohtsuka et al. 2002; Schoch et al. 2002; Kaeser et al. 2011). Essential for short- and long-term synaptic plasticity by affecting the readily releasable pool of vesicles (Castillo et al. 2002, 2002; Blundell et al. 2010; Deng et al. 2011; Han et al. 2011), Rim proteins are also essential for proper targeting of Ca^{2+} channels to presynaptic terminals (Han et al. 2011) and efficient neurotransmitter release (Schoch et al. 2006). Although biochemical studies using native synapstosome membrane preparations failed to demonstrate the existence of a Ca^{2+} channel/Rim complex (Wong and Stanley 2010), various studies report in vitro bindings of Rim with Ca^{2+} channel components. Indeed, direct interaction of Rim-1 with the *synprint* site of $Ca_V2.2$ channels has been shown (Coppola et al. 2001). Moreover, Rim Binding Proteins directly interact with $Ca_V2.2$ channels (and likely with $Ca_V2.1$ channels), possibly providing a molecular link between Ca^{2+} channels and Rim proteins (Hibino et al. 2002). Finally, biochemical interaction of Rim-1 with $Ca_V\beta$ subunits has been reported, slowing $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ channel inactivation when coexpressed in heterologous systems, thereby increasing Ca^{2+} influx during trains of action potentials (Kiyonaka et al. 2007), and a mutation in Rim-1 (R655H) associated with an autosomal dominant cone-rod dystrophy was found to alter Rim-1-dependent modulation of $Ca_V2.1$ channels gating (Miki et al. 2007) leading to a progressive loss of photoreceptors along with retinal degeneration (Barragan et al. 2005; Michaelides et al. 2005). Altogether, these results highlight the critical role of Rim-1 in the modulation of Ca^{2+} homeostasis at nerve terminals.

3.4.4 Cysteine String Proteins (CSP)

Cysteine String Proteins (CSP) are vesicle-associated protein with a key chaperone role at the synapse (Chamberlain and Burgoyne 2000). It was proposed that CSP may serve as a link between $Ca_V2.2$ channels and presynaptic vesicles (Mastrogiacomo et al. 1994). Indeed, CSP interacts with the *synprint* motif of $Ca_V2.1$ (Leveque et al. 1998; Seagar et al. 1999; Magga et al. 2000) and $Ca_V2.2$ channels (Magga et al. 2000). Moreover CSP promotes presynaptic Ca^{2+} influx by recruiting dormant Ca^{2+} channels (Chen et al. 2002). Although the molecular mechanism by which CSP promotes channel activity remains unknown, considering that CSP interacts with syntaxin-1A (Nie et al. 1999; Wu et al. 1999), it is possible that binding of CSP onto syntaxin-1A prevents syntaxin-1A-dependent channel inhibition. Hence, like synaptotagmin-1, CSP may act as a molecular channel switch activity between undocked and docked vesicles release for timely control or presynaptic Ca^{2+} influx.

3.4.5 Huntingtin

Huntingtin (Htt) is well known for its implication in Huntington's disease (Ross and Tabrizi 2011) but the exact cellular function of the protein remains unclear. However, the observation that genetic ablation of Htt is lethal in mice highlights the fundamental importance of the protein (Nasir et al. 1995). Besides interacting with numerous proteins (to date at least 20 proteins involved in gene transcription, cellular transport or cell signaling has been shown to interact with Htt), Htt directly binds to the *synprint* domain of $Ca_V2.2$ channels (Swayne et al. 2005). However, as synaptotagmin-1, coexpression of Htt with $Ca_V2.2$ channels has no consequence on channel gating, but prevents syntaxin-1A-dependent regulation (Swayne et al. 2005), likely by displacing binding of syntaxin-1A from the channel (Swayne et al. 2006). Hence, Htt is not a direct channel modulator per se, but might represent an important actor of synaptic activity by influencing SNARE modulation. However, it remains unclear if Htt is permanently expressed at the synapse under normal condition, or if it is specifically targeted under particular physiopathological states.

3.5 Modulation of Calcium Channels by Other Signaling Pathways

3.5.1 G-Protein Coupled Receptors

Ca^{2+} entry into presynaptic terminals is also modulated by the activation of numerous G-protein-coupled receptors (GPCRs) and second messengers (Jarvis and Zamponi 2001b). Indeed, activation of specific GPCRs following the liberation of

neurotransmitters initiates a negative feedback regulation on presynaptic VGCCs, inhibiting presynaptic Ca^{2+} entry thus terminating synaptic transmission (Brown and Sihra 2008). This spatially delimited regulation (Forscher et al. 1986) relies on the direct binding of free G-protein βγ dimers release upon GPCR activation (Herlitze et al. 1996; Ikeda 1996) to specific intracellular regions of the channel (De Waard et al. 1997, 2005; Zamponi et al. 1997; Tedford and Zamponi 2006). Interestingly, it was shown that cleavage of syntaxin-1A by the botulinium neurotoxin C1 in chick calyx synapses prevents G-protein-dependent inhibition of Ca^{2+} currents, suggesting the involvement of syntaxin-1A in presynaptic G-protein regulation of Ca^{2+} channels (Stanley and Mirotznik 1997; Silinsky 2005). In vitro studies have later revealed an interaction of syntaxin-1A with G-protein βγ dimers, and although syntaxin-1A is not critical for G-protein regulation it potentiates the inhibition in a receptor-independent manner (Jarvis et al. 2000; Jarvis and Zamponi 2001a; Lu et al. 2001). Moreover, G-protein βγ dimers not only interact with syntaxin-1A, but also with SNAP-25 to mediate presynaptic inhibition (Gerachshenko et al. 2005). Finally, besides to modulate Ca^{2+} channel activity, it was shown that G-protein βγ dimers and synaptotagmin-1 compete for binding to the core SNARE complex in a Ca^{2+}-dependent manner such that at high Ca^{2+} concentration synaptotagmin-1 can displace Gβγ binding (Yoon et al. 2007). Hence, Ca^{2+} elevation in presynaptic terminals may prevent G-protein inhibition, likely by preventing binding of Gβγ with SNARE proteins (Yoon et al. 2007). To add further complexity, it was recently reported that Rim-1 promotes relief of G-protein inhibition of $Ca_V2.2$ channels by modulating channel inactivation (Weiss et al. 2011). Altogether, these results highlight the extreme interplay between GPCR-dependent regulation and the molecular actors of the exocytosis process to fine tune presynaptic Ca^{2+} entry.

3.5.2 Phosphorylation

In vitro studies have shown that the protein kinase C (PKC), as well as the Ca^{2+}-calmodulin-dependent kinase II (CaM-KII) are able to phosphorylate the *synprint* domain of $Ca_V2.2$ channels (Yokoyama et al. 1997, 2005), preventing binding of syntaxin-1A and SNAP-25 (Yokoyama et al. 1997) and thus preventing SNARE-dependent inhibition of the channel (Jarvis and Zamponi 2001a). However, uncoupling of SNARE proteins from the channel upon *synprint* phosphorylation, most likely represent a termination signal for synaptic exocytosis. It was reported that phosphorylation of the *synprint* site of $Ca_V2.1$ channels by the glycogen synthase kinase-3 (GSK-3) prevents SNARE interaction with the channel but also inhibits synaptic exocytosis possibly by interfering with Ca^{2+} channel/SNARE coupling (Zhu et al. 2010). Interestingly, phosphorylation of syntaxin-1A and SNAP-25 by PKC or CaM-KII does not alter interaction with the *synprint* site (Yokoyama et al. 1997), Hence, PKC- and CaM-KII-dependent phosphorylation of

the *synprint* site may serve as a biochemical switch for interaction/modulation of voltage-gated Ca^{2+} channels with SNARE protein complexes.

3.6 Concluding Remarks

Like most key cellular functions, control of neurotransmitter release by presynaptic Ca^{2+} channels is highly regulated. The various components of the exocytosis machinery, besides localizing the vesicles within the vicinity of the source of Ca^{2+}, provide a potent reciprocal control of presynaptic Ca^{2+} influx by modulating channel gating. This dynamic regulation appears to be fundamental to dynamically and temporally fine tune neurotransmitter release. Surprisingly, as highlighted in this chapter, the regulation of presynaptic Ca^{2+} channels appears extremely complex, with intricate interplay between different types of synaptic proteins and second messenger signaling pathways, but also highly redundant. This important redundancy in Ca^{2+} channel regulation by various presynaptic proteins might ensure a security control over a fundamental physiological function. Finally, although $Ca_V2.1$/$Ca_V2.2$ and $Ca_V3.2$ T-type channels use completely distinct channel molecular determinants to interact with the vesicular machinery, they are functionally regulated by syntaxin-1A and SNAP-25 in a strikingly similar manner. This may perhaps underscore the fundamental importance of localizing the exocytosis machinery near the source of Ca^{2+}, and providing tight control over Ca^{2+} entry.

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Chapter 4
Molecular Architecture of Ca^{2+} Channel Complexes Organized by $Ca_V\beta$ Subunits in Presynaptic Active Zones

Akito Nakao, Mitsuru Hirano, Yoshinori Takada, Shigeki Kiyonaka, and Yasuo Mori

Abstract Fine regulation of neurotransmitter release at presynaptic active zones is crucial for nervous system adaptive functions, including learning, memory and cognition. Neurotransmitter release is controlled by Ca^{2+} influx into the presynaptic active zones via voltage-gated Ca^{2+} channels (VGCCs). Therefore, the molecular organization of VGCC complexes at the active zones is important for Ca^{2+}-triggered neurotransmitter release. Through rigorous investigation about VGCC complexes at active zones, it is becoming clear that active zone proteins impact functionally on VGCCs through direct or indirect interaction with $Ca_V\alpha_1$ and/or $Ca_V\beta$ subunits. Specifically, Rab3-interacting molecules (RIMs), which modulate the small G protein Rab3 that is involved in synaptic vesicle trafficking, have been identified in VGCC complexes. In this review, we mainly discuss active zone proteins that form complexes with VGCCs through $Ca_V\beta$ subunits and how these proteins regulate the function of VGCCs in neurotransmitter release.

Keywords Active zone proteins • $Ca_V\beta$ subunit • RIM • SM protein • SNAREs

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4.1 Introduction

Fine regulation of presynaptic active zone neurotransmitter release is integral to nervous system adaptive functions, including learning, memory and cognition. The molecular organization of presynaptic active zones, where synaptic vesicles are docked in close vicinity to voltage-gated Ca^{2+} channels (VGCCs) at the presynaptic membrane, is essential for controlling the neurotransmitter release triggered by depolarization-induced Ca^{2+} influx (Neher 1998).

The presynaptic active zone is a specialized site for neurotransmitter release in the nerve terminals, and is characterized by its high-electron density when viewed using electron microscopy (Landis et al. 1988; Zhai and Bellen 2004; Atwood 2006). Recent biochemical and molecular biological approaches have identified active zone scaffold proteins including CAST/ELKS (Ohtsuka et al. 2002; Wang et al. 2002; Deguchi-Tawarada et al. 2004), Bassoon (tom Dieck et al. 1998), Piccolo/Aczonin (Wang et al. 1999; Fenster et al. 2000), Munc13-1 (Brose et al. 1995) and Rab3-interacting molecules (RIMs) (Wang et al. 1997). The architecture of the active zone is exquisitely designed to regulate the release of neurotransmitters.

The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and the Sec1/Munc18 (SM) protein, Munc18, have emerged as probably the pivotal components of the exocytotic apparatus, and elegant models of their functions have been developed (Rizo and Südhof 2002). SNAREs putatively generate specificity in membrane fusion and directly execute fusion by forming a tight complex that brings the synaptic vesicle and plasma membrane together (Weber et al. 1998). Munc18 has been suggested to assist in the formation of this complex, and models of the roles of other presynaptic proteins generally revolve around their interactions with SNAREs and/or Munc18. Recently, it has been suggested that Munc18 influences all processes leading to exocytosis, including vesicle recruitment, tethering, docking, priming and membrane fusion (Burgoyne et al. 2009).

In impulse-evoked neurotransmitter release, Ca^{2+} influx into presynaptic active zones via VGCCs is an essential step (Takahashi and Momiyama 1993; Wheeler et al. 1994; Catterall 1998). Physical and functional coupling of VGCCs to the vesicular release machinery, including SNAREs and SM protein, is critical for efficient neurotransmitter release (Stanley 1993; Sheng et al. 1994; Bezprozvanny et al. 1995; Neher 1998; Chan et al. 2007; Wadel et al. 2007; Catterall and Few 2008). It was originally believed that the anchorage of Ca^{2+} channels close to the Ca^{2+} microdomain-dependent release machinery was the main reason for the physical interactions between these channels and synaptic proteins (Sheng et al. 1994). In addition, it is now becoming clear that associated proteins additionally regulate channel activity.

Recently, it has been reported that, like SNAREs and the SM protein, active zone scaffold proteins interact physically and functionally with VGCCs (Kiyonaka et al. 2007; Uriu et al. 2010; Chen et al. 2011; Kiyonaka et al. 2012). Among the active

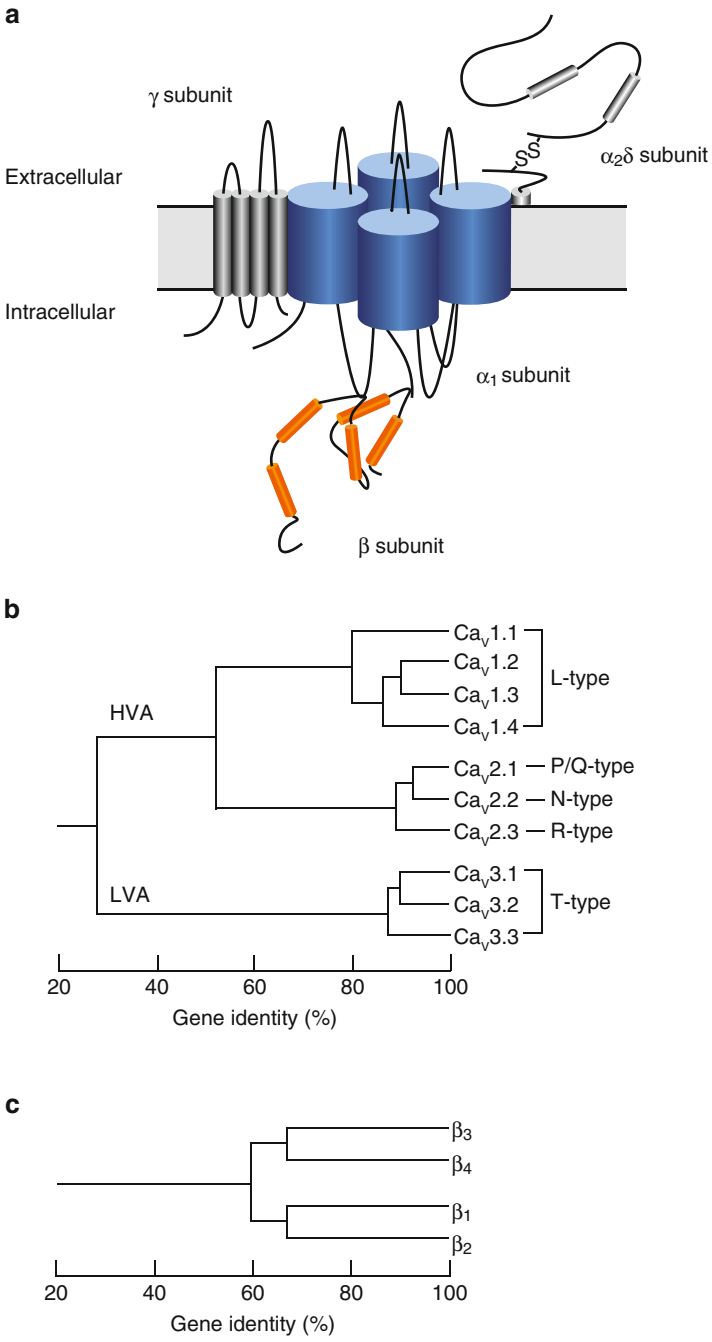
zone scaffold proteins, the RIMs are attracting increasing attention because of their apparently crucial role in the localization and regulation of VGCCs. This paper aims to review recent advances in our understanding of VGCC complexes and their possible physiological significance in the active zone.

4.2 Voltage-Gated Ca^{2+} Channels

Several types of VGCCs, distinguished on the basis of their biophysical and pharmacological properties, coexist in neurons (Tsien et al. 1991). VGCCs are heteromultimeric protein complexes composed of the pore-forming α_1 subunit (designated as Ca_V) and auxiliary $\alpha_2\delta$, β and γ subunits (Ertel et al. 2000) (Fig. 4.1a). The $Ca_V\alpha_1$ subunit consists of about 2,000 amino acid residues containing four homologous repeats (I–IV), each of which contains six transmembrane segments (S1–S6) and a membrane-associated loop between S5 and S6 (Fig. 4.2) (Catterall 1998, 2000, 2011; Catterall and Few 2008; Buraei and Yang 2010). The four S5–S6 loops (one for each homologous repeat) form the ion-selectivity filter, which is essential for the selective conductance of Ca^{2+} (Kim et al. 1993; Kuo and Hess 1993; Yang et al. 1993; Sather and McCleskey 2003). Positively charged amino acids in the S4 segments form the voltage sensor. The $Ca_V\alpha_1$ subunit is encoded by ten distinct genes, whose roles in various functional types have been largely elucidated (Tsien et al. 1991; Ertel et al. 2000). Based on amino acid sequence similarity, $Ca_V\alpha_1$ subunits are divided into three subfamilies: Ca_V1, Ca_V2, and Ca_V3 (Fig. 4.1b) (Catterall 2000; Ertel et al. 2000; Arikkath and Campbell 2003; Yang and Berggren 2006; Buraei and Yang 2010). Channels in the Ca_V1 subfamily conduct L-type Ca^{2+} currents; the Ca_V2 subfamily includes channels that conduct N-, P/Q-, and R-type Ca^{2+} currents; and the Ca_V3 subfamily channels conduct T-type Ca^{2+} currents (Fig. 4.1b) (Ertel et al. 2000). N-, P/Q-, R- and L-type Ca^{2+} currents are essential for neurotransmitter release (Takahashi and Momiyama 1993; Wheeler et al. 1994; Catterall 1998).

Members of the Ca_V1 and Ca_V2 families associate with auxiliary β subunits, α_2/δ subunits and γ subunits (Fig. 4.1a) (Catterall 1998, 2000, 2011 Catterall and Few 2008; Buraei and Yang 2010). These auxiliary subunits modulate the membrane expression and biophysical characteristics of the $Ca_V\alpha_1$ subunit (Arikkath and Campbell 2003; Kang and Campbell 2003; Klugbauer et al. 2003).

$Ca_V\alpha_2\delta$ subunits are encoded by four different genes and can modify channel biophysical properties (Singer et al 1991; Wakamori et al 1994; Cantí et al. 2003;), although their primary role is to increase Ca^{2+} channel current (Mikami et al. 1989; Mori et al. 1991; Singer et al. 1991; Wakamori et al 1994; Klugbauer et al. 1999; Gao et al. 2000; Cantí et al. 2003; Klugbauer et al. 2003; Davies et al. 2006) by promoting trafficking of the $Ca_V\alpha_1$ subunit to the plasma membrane and/or by increasing its retention there (Gurnett et al. 1997; Sandoval et al. 2004; Cantí et al. 2005; Bernstein and Jones 2007).


a
γ subunit
Extracellular
Intracellular
S-S
α2δ subunit
α1 subunit
β subunit
b
HVA
LVA
CaV1.1
CaV1.2
CaV1.3
CaV1.4
L-type
CaV2.1
CaV2.2
CaV2.3
P/Q-type
N-type
R-type
CaV3.1
CaV3.2
CaV3.3
T-type
20
40
60
80
100
Gene identity (%)
c
β3
β4
β1
β2
20
40
60
80
100
Gene identity (%)

It has been reported that the $Ca_V\gamma_2$ subunit (stargazin) was the target of the stargazer mutation in mice (Letts et al. 1998); a related series of seven $Ca_V\gamma$ subunits is expressed in brain and other tissues (Klugbauer et al. 2000). $Ca_V\gamma_{1-4}$ subunits have various effects on VGCC activity, depending on the combination of $Ca_V\alpha_1$ and $Ca_V\beta$ subunits with which they are complexed (Singer et al. 1991; Wei et al. 1991; Eberst et al. 1997; Letts et al. 1998; Freise et al. 2000; Klugbauer et al. 2000; Kang et al. 2001; Rousset et al. 2001; Held et al. 2002). These $Ca_V\gamma$ subunits are not only auxiliary subunits of VGCCs, but are also the primary modulators of glutamate receptors in the postsynaptic membranes of brain neurons (Nicoll et al. 2006). Therefore, γ subunits are also called transmembrane AMPA receptor regulatory proteins. Interestingly, specific association of $Ca_V2.1$ and $Ca_V2.2$ with AMPA receptors in the postsynaptic membrane has been reported (Kang et al. 2006).

Two roles of $Ca_V\beta$ subunits, which are VGCC auxiliary subunits encoded by four different genes (Fig. 4.1c), have been proposed: (1) enhancement of functional expression (Mori et al. 1991) and (2) a direct effect on gating (Lacerda et al. 1991; Varadi et al. 1991). With respect to their effect on expression, the interaction between $Ca_V\beta$ and $Ca_V\alpha_1$ subunits may enhance channel trafficking to the plasma membrane by masking an endoplasmic reticulum retention signal in the $Ca_V\alpha_1$ subunit (Mori et al. 1991; Bichet et al. 2000; Fang and Colecraft 2011) and/or protecting the channel complex from proteosomal degradation (Altier et al. 2011; Waithe et al. 2011). The $Ca_V\beta$ subunits have also been found to regulate the voltage dependence of activation and increase the channel open probability, thus increasing current through individual channels and resulting in augmented macroscopic current density (Lacerda et al. 1991; Varadi et al. 1991; Walker and De Waard 1998; Dolphin 2003; Buraei and Yang 2010).

The $Ca_V\beta$ and $Ca_V\alpha_2\delta$ subunits are traditionally considered to be auxiliary subunits that enhance channel trafficking, increase the functional expression of VGCCs at the plasma membrane and influence the biophysical properties of VGCCs. Accumulating evidence indicates that these subunits may also have roles in the nervous system that are not directly linked to Ca^{2+} channel function (Dolphin 2012). For instance, $Ca_V\beta$ subunits work as transcriptional regulators (Hibino et al. 2003; Zhang et al. 2010; Xu et al. 2011; Tadmouri et al. 2012), and certain $Ca_V\alpha_2\delta$ subunits may function in synaptogenesis (Eroglu et al. 2009).

Fig. 4.1 (**a**) The subunit composition of high-voltage activated (HVA) VGCCs. (**b**) The phylogenetic tree of $Ca_V\alpha_1$ subunits which can be divided into three subclasses according to gene identity. The Ca_V1 and Ca_V2 classes are HVA channels. The Ca_V1 subfamily form channels which conduct L-type Ca^{2+} currents; The Ca_V2 subfamily form channels which conduct N-, P/Q-, and R-type Ca^{2+} currents. The Ca_V3 class is low-voltage activated (LVA) channels. The Ca_V3 subfamily form channels which conduct T-type Ca^{2+} currents. (**c**) The phylogenetic tree of $Ca_V\beta$ subunits. There are four subfamilies of $Ca_V\beta$ subunits ($Ca_V\beta_1$-β_4) encoded by four distinct genes

4.3 VGCC Complexes at Presynaptic Active Zones

Neurons have high buffering capacity, and thus free Ca^{2+} is thought to diffuse only short distances before being sequestered by Ca^{2+} sensor proteins such as synaptotagmin (Augustine 2001). Hence, to sense Ca^{2+} entry, VGCCs must be localized in close proximity to the vesicular release machinery (Neher 1998; Spafford and Zamponi 2003). For this reason, the physical and functional coupling between VGCCs and active zone proteins (Fig. 4.2) enhances the efficiency of neurotransmission.

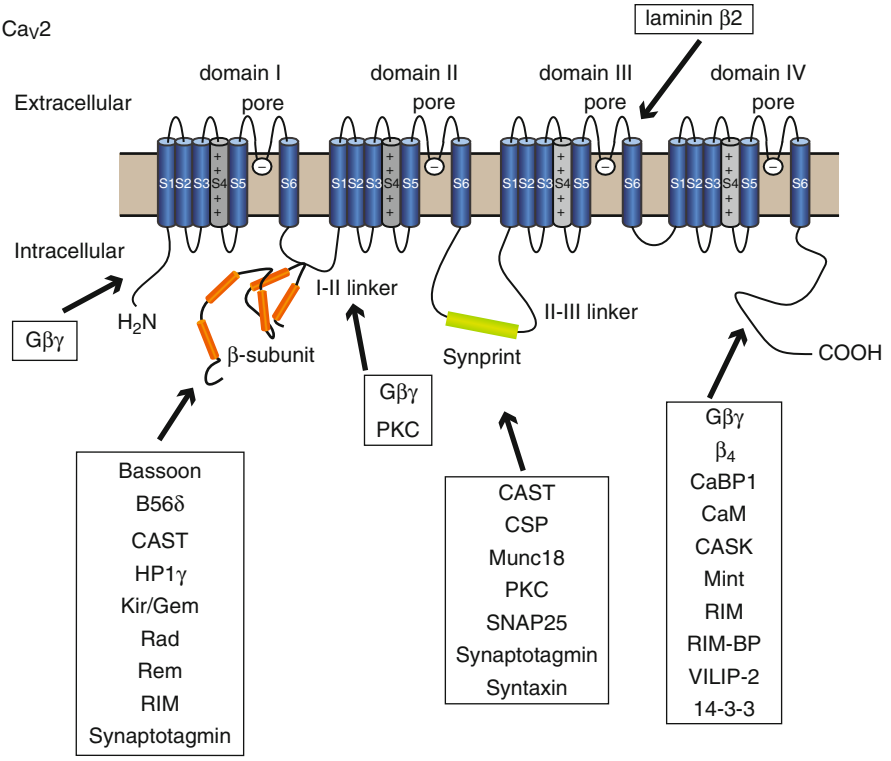


Fig. 4.2 The presynaptic VGCC signaling complexes. Regulatory proteins and their interaction site on VGCCs are illustrated. Gβγ subunits bind to N-terminal of $Ca_V\alpha_1$ subunits. Gβγ subunits and protein kinase C (PKC) (Zamponi et al. 1997) bind to I-II linker of $Ca_V\alpha_1$ subunits. CAST, cysteine string protein (CSP) (Leveque et al. 1998), Munc18, PKC (Yokoyama et al. 1997), SNAP25, synaptotagmin and syntaxin bind to II-III linker (the synprint site) of $Ca_V\alpha_1$ subunits. Laminin β2 binds to the extracellular loop of $Ca_V\alpha_1$ subunits. Gβγ subunits, VGCC β_4 subunit, Ca^{2+} binding protein 1 (CaBP1), CaM, CASK, Mint, RIM, RIM-BP, visinin like protein 2 (VILIP-2) (Nanou et al. 2012) and 14-3-3 (Li et al. 2006) bind to C-terminal of $Ca_V\alpha_1$ subunits. Bassoon, B56δ (Tadmouri et al. 2012), CAST, HP1γ, Kir/Gem, Rad, Rem, RIM and synaptotagmin bind to $Ca_V\beta$ subunits

Previously much research on the interaction of VGCCs focused on the $Ca_V\alpha_1$ subunit, which contains the Ca^{2+}-selective filter and voltage sensor. For example, CASK and Mint1 associate with the C-terminal of the $Ca_V\alpha_1$ subunit (Fig. 4.2) in hippocampal neurons (Maximov et al. 1999; Maximov and Bezprozvanny 2002). It is notable that the extracellular protein, laminin, interacts with VGCCs via the extracellular loop of the $Ca_V\alpha_1$ subunit (Fig. 4.2) in motor nerve terminals (Nishimune et al. 2004). The $Ca_V\alpha_1$ subunit also contains sequences in the cytoplasmic II–III loop, termed the synaptic protein interaction (synprint) site, which are particularly important for interaction with the synaptic vesicle fusion apparatus, including SNAREs and SM protein (see below for detail) (Fig. 4.2).

Recently, $Ca_V\beta$ subunits have been mooted as the platform of VGCC complexes because they contain an SH3-HOOK-GK module that places them within a family of proteins called the membrane-associated guanylate kinases (MAGUKs) (Buraei and Yang 2010). In general, MAGUKs (e.g. PSD95, SAP97, CASK, Shank and Homer) function as scaffold molecules that play a key role in organizing multiprotein complexes at functionally specialized regions such as synapses and other cellular junctions (Takahashi et al. 2004; Funke et al. 2005; Elias and Nicoll 2007). It has been reported that these $Ca_V\beta$ subunits interact with various proteins (Fig. 4.2), including small G-proteins such as Kir/Gem, Rem and Rad that directly interact with the $Ca_V\beta$ subunit to regulate VGCC activity (Béguin et al. 2001; Finlin et al. 2003). The $Ca_V\beta_{4c}$ subunit interacts with heterochromatin protein 1γ (HP1γ), a nuclear protein involved in gene silencing and transcriptional regulation (Hibino et al. 2003; Xu et al. 2011). The function of $Ca_V\beta$ subunits as a platform for VGCC complexes is believed to occur at the presynaptic active zones (Fig. 4.2) (Kiyonaka et al. 2007, 2012; Uriu et al. 2010; Chen et al. 2011), which is critical for VGCCs and active zone proteins to couple functionally to regulate neurotransmitter release.

In the following section, we focus on VGCC complexes formed by the SNAREs, SM protein and active zone scaffold proteins through interaction with $Ca_V\alpha_1$ and/or $Ca_V\beta$ subunits. Among active zone scaffold proteins, RIMs are a fascinating binding partner for VGCCs, and will be discussed in some detail in this chapter.

4.3.1 Functional Interaction of VGCCs with SNAREs and/or SM Protein

Ca^{2+} entry through VGCCs initiates neurotransmitter release by triggering the fusion of secretory vesicle membranes with the plasma membrane through the actions of SNAREs and SM protein (Bajjalieh and Scheller 1995; Südhof and Rizo 2011). Previous reports have demonstrated the functional impact of syntaxin, synaptosome-associated protein of 25 kDa (SNAP-25) and synaptotagmin on VGCCs through their physical association with the synprint region in the II-III linker of the α_1 subunit (Fig. 4.2) (Bezprozvanny et al. 1995; Wiser et al. 1996; Charvin et al. 1997; Sheng et al. 1997; Zhong et al. 1999; Spafford and Zamponi

2003). Coexpression of syntaxin or SNAP-25 with $Ca_V2.1$ or $Ca_V2.2$ channels reduces the level of channel expression and inhibits Ca^{2+} channel activity by shifting the voltage dependence of steady-state inactivation during long depolarizing prepulses toward more negative membrane potentials (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999). These inhibitory effects are relieved by the formation of a complete SNARE complex containing syntaxin and SNAP-25 (Wiser et al. 1997; Tobi et al. 1998; Zhong et al. 1999), allowing rapid activation of the Ca_V2 channels that would not be possible in the absence of syntaxin and SNAP-25 because of the desensitization characteristics of these channels. Recently, it has been reported that endogenous SNAP-25 negatively regulates native VGCCs in glutamatergic neurons (Condliffe et al. 2010), which could have important implications for neurological diseases that are associated with decreased SNAP-25 expression, such as schizophrenia and epilepsy (Thompson et al. 2003; Zhang et al. 2004). Furthermore, the functional modulation of VGCC by Munc18-1 and the direct binding of Munc18-1 to the synprint site of the $Ca_V\alpha_1$ subunit have been shown (Fig. 4.2) (Chan et al. 2007), although the causal relationship between these two phenomena has yet to be demonstrated definitively. Thus, SNAREs and SM protein differentiate VGCCs within active zones from those outside.

4.3.2 Functional Interaction of VGCCs with RIM Active Zone Scaffold Protein via $Ca_V\beta$ Subunits

The presynaptic active zones consist of a dense accumulation of cytomatrix proteins, including CAST/ELKS, Bassoon, Piccolo/Aczonin, Munc13-1, α-liprin, RIM-binding protein (RIM-BP) and RIMs. RIMs are among the most interesting of these scaffold proteins because, as VGCC binding partners, they are central organizers of the active zones. Originally identified as a putative effector of the synaptic vesicle protein Rab3 (Wang et al. 1997), RIM1α is part of the RIM superfamily, whose members share a common C_2B domain at their C terminal (Wang and Südhof 2003). With regard to RIM1 and RIM2, a long isoform (α) and two short isoforms (β and γ, that lack the Rab3-interacting Zn^{2+} finger domain) are known, whereas only short γ forms are known for RIM3 and RIM4 (Wang and Südhof 2003; Kaeser et al. 2008). RIMs are central organizers of active zones because they directly or indirectly interact with other presynaptic active zone protein components, including Munc13, CAST/ELKS, RIM-BP, and α-liprin, to form a protein scaffold in the presynaptic nerve terminal (Wang et al. 2000; Betz et al. 2001; Coppola et al. 2001; Ohtsuka et al. 2002; Schoch et al. 2002). It has been shown that RIM1α is essential for different forms of synaptic plasticity in different types of synapse (Castillo et al. 2002; Schoch et al. 2002; Fourcaudot et al. 2008). More recently, deletion of RIM1α and RIM1β in mice was reported to severely impair survival (Kaeser et al. 2008). Electrophysiological analyses showed that RIM1α knockout was sufficient to completely abolish long-term presynaptic

plasticity in mice, with the additional knockout of RIM1β having no further effect (Kaeser et al. 2008). In contrast, the impairment of synaptic strength and short-term synaptic plasticity that is caused by RIM1α deletion was not complete and was aggravated by the additional deletion of RIM1β, suggesting that RIM1α and RIM1β have distinct but overlapping functions (Kaeser et al. 2008). Mice deficient in both RIM1α and RIM2α showed lethality due to defects in Ca^{2+}-triggered release, despite normal presynaptic active zone length and normal spontaneous neurotransmitter release (Schoch et al. 2006). In *Caenorhabditis elegans*, the loss of the single RIM homolog, UNC10, causes a reduction in membrane-contacting synaptic vesicles within 30 nm of the dense projection at neuromuscular junctions (Weimer et al. 2006).

We showed that RIM1α associated with $Ca_V\beta$ subunits via its C_2B domain-containing C terminus to markedly suppress voltage-dependent inactivation in various neuronal VGCCs (Kiyonaka et al. 2007). Consistent with this, acetylcholine release in PC12 pheochromocytoma neuroendocrine cells was significantly potentiated by full-length and C-terminal RIM1α constructs, but membrane docking of vesicles was enhanced only by the full-length RIM1α. The $Ca_V\beta$-AID dominant negative $Ca_V\beta$ subunit construct, which disrupts the RIM1α-$Ca_V\beta$ association, accelerated the inactivation of native VGCC currents, suppressed vesicle docking and acetylcholine release in PC12 cells, and inhibited glutamate release in cultured cerebellar neurons. We therefore propose that the interaction of RIM1α with $Ca_V\beta$ subunits in the presynaptic active zone supports neurotransmitter release via two distinct mechanisms: (1) by sustaining Ca^{2+} influx through inhibition of voltage-dependent VGCC inactivation and (2) by anchoring neurotransmitter-containing vesicles in the vicinity of VGCCs (Figs. 4.3 and 4.4). In support of our hypothesis regarding VGCC-RIM1α association, a report states that RIM colocalizes with Ca^{2+} channels in *C. elegans*, facilitating vesicle targeting to the presynaptic density through direct interaction with Rab3 (Gracheva et al. 2008).

Recently, Gandini et al. have reported that the coupling between RIM1 and $Ca_V\beta$ subunits was important for decelerating the inactivation kinetics of L-type VGCCs in RIN-m5F cells, an insulin-secreting cell line (Gandini et al. 2011). Likewise, Gebhart et al. demonstrated that the presence of $Ca_V\beta$ subunits is necessary for the interaction of RIM proteins with the $Ca_V1.3$ channel complex and the subsequent deceleration of $Ca_V1.3$ channel inactivation in tsA-201 cells heterologously expressing these channels (Gebhart et al. 2010). The same report also demonstrated that RIM proteins are expressed in cochlear inner hair cells (Gebhart et al. 2010), in which high levels of VGCCs are functionally expressed. Indeed, RIM2α co-localized with $Ca_V1.3$ in the same presynaptic compartment of these inner hair cells (Gebhart et al. 2010). These reports are consistent with our idea that $Ca_V\beta$ subunits are essential for the regulation of VGCCs by RIMs.

Interestingly, Ca_V2 current inactivation is also markedly decelerated by γ-RIMs, which have only the C_2B domain, suggesting that suppression of inactivation kinetics is a common feature of RIM regulation of VGCC currents (Uriu et al. 2010; Kaeser et al. 2012). In PC12 cells, this common functional feature allows native RIMs to enhance acetylcholine secretion, whereas γ-RIMs are uniquely different

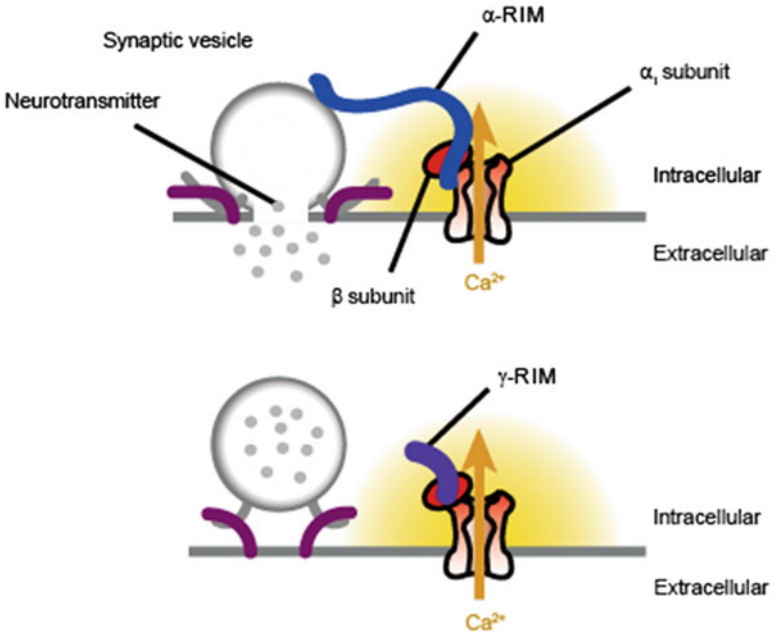


Fig. 4.3 Functional coupling between VGCCs and RIMs. α-RIMs anchor synaptic vesicles next to channels and maintain depolarization-triggered Ca^{2+} influx. On the other hand, γ-RIMs only maintain depolarization-triggered Ca^{2+} influx but can't anchor vesicles

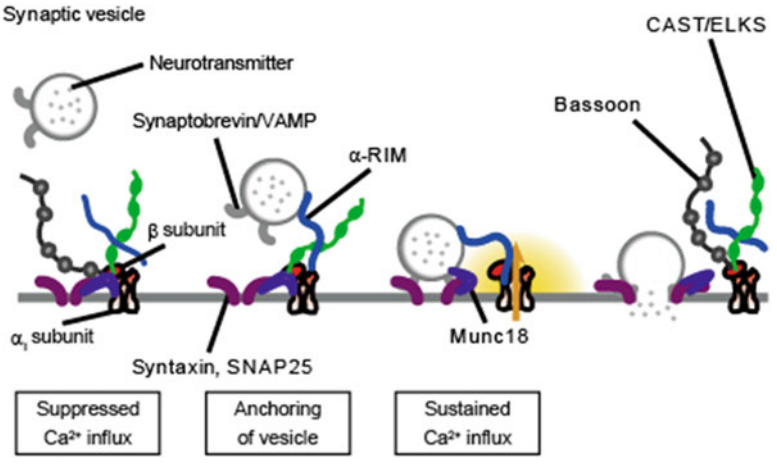


Fig. 4.4 Putative model of dynamic regulation of VGCCs properties at presynaptic active zone. α-RIMs anchor synaptic vesicles next to channels through its interaction with active zone specific proteins and the $Ca_V\beta$ subunit. After depolarization, RIMs suppress inactivation of VGCCs, which result in a sustained Ca^{2+} influx. These molecular interactions support neurotransmitter release

from α-RIMs in blocking localization of neurotransmitter-containing vesicles near the plasma membrane. γ- and α-RIMs are diffusely distributed in central neurons, but knockdown of γ-RIMs attenuated glutamate release to a lesser extent than that of α-RIMs in cultured cerebellar neurons (Uriu et al. 2010). Thus, it appears that Ca^{2+} influx sustained by suppression of VGCC inactivation by RIMs is a ubiquitous property of neurons, whereas the extent of vesicle anchoring to VGCCs at the plasma membrane may depend on competition between α- and γ-RIMs for $Ca_V\beta$ subunits (Fig. 4.3).

Furthermore, we demonstrated that a mouse RIM1α arginine-to-histidine substitution (R655H), which corresponds to the human autosomal dominant cone-rod dystrophy mutation (Johnson et al. 2003), modifies the ability of RIM1α to regulate VGCC currents elicited by the P/Q-type $Ca_V2.1$ and L-type $Ca_V1.4$ channels (Miki et al. 2007). Recently, it has been shown that the *RIM3* gene is a novel candidate for autism (Kumar et al. 2010). The variants identified in autism patients are located in the domain that interacts with the $Ca_V\beta$ subunit, which may impair the function of RIM3 in relation to VGCC in autism patients.

More recently, it has been reported that RIMs also bind to the C-termini of N- and P/Q-type VGCCs through a PDZ-domain (Fig. 4.2) (Kaeser et al. 2011; Südhof 2012). The binding of the RIM PDZ-domain to VGCCs is important for recruiting VGCCs to active zones (Han et al. 2011; Kaeser et al. 2011; Südhof 2012). Synapses expressing a mutant RIM that lacks the PDZ-domain exhibit a selective loss of VGCCs, resulting in an elevated Ca^{2+}-dependent release threshold and a desynchronization of release (Kaeser et al. 2011). In addition to binding directly to RIMs, VGCCs are bound indirectly via RIM-BPs (Hibino et al. 2002) thus tethering VGCCs to the active zone. Specifically, the SH3-domains of RIM-BPs interact with a PXXP motif in both RIMs (localized between the C_2A and C_2B domains) and VGCCs (in their cytoplasmic tails). A RIM fragment consisting of only its PDZ domain and the PXXP motif is sufficient to rescue the presynaptic loss of VGCCs in RIM-deficient synapses (Kaeser et al. 2011). Together, these data suggest that VGCCs are recruited to active zones by a complex composed of RIMs, RIM-BPs, and VGCC C-termini (Südhof 2012).

G-protein-coupled receptor (GPCR)-mediated inhibition is one of the most important modes of VGCC regulation (Zamponi 2001; Strock and Diversé-Pierluissi 2004). This inhibition by GPCRs is based on the direct binding of the Gβγ signaling complex to the Ca_V2 pore-forming subunit (Fig. 4.2) (De Waard et al. 1997; Zamponi et al. 1997). Facilitation of Ca^{2+} influx via VGCCs may also be caused by abrogating this inhibitory GPCR pathway. Weiss et al. explored the functional implication of RIM1 in G-protein regulation of N-type VGCCs using a recombinant expression system (Weiss et al. 2011). Activation of μ-opioid receptors co-expressed in HEK-293 cells with N-type VGCCs (but without RIM1) produced a dramatic inhibition of both the initial and sustained current. In contrast, the additional expression of RIM1, while having no effect on the initial current through the channel, considerably reduced the GPCR-mediated inhibitory

effect in the subsequent phases of channel activity, favoring sustained Ca^{2+} influx during prolonged activity. Thus, RIM1 may facilitate neurotransmitter release by promoting the recovery of the channel from G-protein-mediated inhibition. This contributes, together with the slowing of channel inactivation, to maintain Ca^{2+} influx under prolonged activity.

In non-neuronal cells, the coupling between VGCCs and RIMs also play important roles. Shibasaki et al. reported that RIM2 directly binds to L-type VGCCs in a GST pull-down assay (Shibasaki et al. 2004). RIM2α was also recently shown, using knockout mice, to be necessary for the docking and priming of K^+-induced insulin granule secretion in pancreatic cells (Yasuda et al. 2010). These experiments also revealed that RIM2α regulates the voltage dependence of VGCC current inactivation in these cells (Yasuda et al. 2010). Gandini et al. found that RIM1 was important for decelerating the inactivation kinetics of L-type VGCCs in RIN-m5F cells (an insulin-secreting cell line), and that K^+-induced insulin release was significantly decreased in RIM1 knockdown cells (Gandini et al. 2011). These reports suggest that coupling between VGCCs and RIMs has key roles in insulin granule exocytosis.

4.3.3 Functional Interaction of VGCCs with Other Active Zone Scaffold Proteins

Another active zone scaffold protein, CAST/ELKS, physically interacts with $Ca_V\beta$ subunits (Fig. 4.2) and functionally modulates the channel activation properties (Chen et al. 2011; Billings et al. 2012; Kiyonaka et al. 2012). CAST/ELKS shifts the voltage-dependency of activation towards hyperpolarized potentials, and thus may regulate neurotransmitter release through the formation of its protein complex with VGCCs and the modulation of their opening (Kiyonaka et al. 2012). It has been also shown that Bruchpilot, the *Drosophila* homolog of mammalian CAST/ELKS, associates with the C-terminus of the $Ca_V\alpha_1$ subunit homolog, Cacophony (Fouquet et al. 2009). Furthermore, Piccolo/Aczonin and Bassoon are large multidomain proteins specific to vertebrates whose major function appears to be to guide synaptic vesicles to the active zone (Südhof 2012). Piccolo/Aczonin and Bassoon are composed of highly homologous zinc finger and coiled-coil sequences so are able to interact with other presynaptic proteins. Previous reports show that Bassoon and Piccolo/Aczonin are found in protein complexes containing VGCCs in vivo (Carlson et al. 2010; Frank et al. 2010). Furthermore, Bassoon interacts with $Ca_V\beta$ subunits (Fig. 4.2) to organize the neuromuscular junction active zone, and suppresses the inactivation of VGCCs (Chen et al 2011; Nishimune et al. 2012). Thus, Bassoon has the potential to modulate synaptic transmission efficiency by interacting with presynaptic VGCC complexes and modifying channel function. Tripartite complexes composed of modular adapter proteins CASK, Mint1 and Veli are present in the presynaptic active zone (Butz et al. 1998; Tabuchi et al. 2002). CASK, which is composed of an N-terminal CaM kinase domain, PDZ and SH3 domains and a C-terminal GK domain, binds to Mint1 through its CaM

kinase domain. It has been reported that CASK and Mint1 associate with the cytosolic C-terminus of the $Ca_V\alpha_1$ subunit and modulate the synaptic targeting of N-type VGCCs in hippocampal neurons (Maximov et al. 1999; Maximov and Bezprozvanny 2002). However, despite considerable data linking CASK and Mint1 to synapses, their precise roles are elusive. These results suggest that VGCCs are essential for anchoring these active zone proteins to the presynaptic membrane during organization of the active zones.

4.4 Dynamic Regulation of VGCC Properties by Presynaptic Active Zone Proteins

Ca^{2+}-triggered neurotransmitter release requires close proximity of docked neurotransmitter-containing synaptic vesicles and VGCCs at presynaptic active zones (Fig. 4.4). VGCCs are recruited to active zones by a complex composed of RIMs, RIM-BPs, and VGCCs (Kaeser et al. 2011). However, syntaxin and SNAP-25 have been proposed to inhibit VGCC-mediated Ca^{2+} influx by inducing a hyperpolarizing shift of the inactivation curve in the absence of vesicle docking at VGCC sites (Bezprozvanny et al. 1995; Spafford and Zamponi 2003). The inhibitory effects of syntaxin are relieved by SNAP-25 and synaptotagmin in the complete SNARE complex (Wiser et al. 1997; Tobi et al. 1998; Zhong et al. 1999). In a nerve terminal, this reversible negative shift in the voltage dependence of inactivation would provide a molecular switch to inhibit VGCCs when associated with an immature SNARE protein complex, but to disinhibit them as the synaptic core complex matures in preparation for vesicle release (Fig. 4.4) (Zhong et al. 1999).

Previous reports suggest that RIM1 is involved in modification of the release apparatus at a late stage in the vesicle cycle (Schoch et al. 2002), particularly in the post-docking step (Koushika et al. 2001). Our findings infer enhancement or maintenance of Ca^{2+} influx via interaction with RIM1α during vesicle docking (Kiyonaka et al. 2007). Taking into consideration the direct RIM1α-Rab3 association and the regulation of tethering and/or priming of synaptic vesicles by Rab3 (Dulubova et al. 2005), it is likely that simultaneous interactions of RIM1α with vesicle-associated Rab3 and Munc13 (via the N-terminal Zn^{2+}-finger domain) and with $Ca_V\beta$ subunits (via the C-terminal C_2B domain) is at least part of the mechanism maintaining close proximity between VGCCs and vesicles, thereby regulating the dynamic properties of synaptic transmission (Neher 1998). Thus, the $Ca_V\alpha_1$ protein associations and the RIM1α-$Ca_V\beta$ association may be distinct interactions that contribute at different stages of vesicle cycling to control the Ca^{2+} supply from the source (i.e. the VGCC) and to regulate the proximity between the Ca^{2+} source (VGCC) and the target Ca^{2+} sensors at the presynaptic active zone. When voltage-dependent inactivation of VGCCs is suppressed by RIMs, the responses of Ca^{2+} sensors, such as synaptotagmins, to depolarizing membrane potentials can be potentiated at presynaptic active zones. Interestingly, specific physiological roles played by γ-RIMs can weaken the physical coupling between

VGCCs and synaptic vesicles (Uriu et al. 2010). We can therefore hypothesize that the identity of the RIM coupling to presynaptic VGCCs (α-RIM or γ-RIM) may determine the tightness of coupling between Ca^{2+} influx and fusion of synaptic vesicles, and could thus dictate the efficacy of synaptic transmission (Fig. 4.3). In addition, RIM1 reduces the inhibitory pathway mediated by GPCRs (Weiss et al. 2011), which may represent another mechanism by which it modulates synaptic transmission.

4.5 Conclusions

Presynaptic VGCCs are associated with various active zone proteins involved in the fusion of transmitter-containing vesicles with the presynaptic membrane. Among these proteins, RIM1 links synaptic vesicles and depolarization-induced Ca^{2+} influx by interacting with Rab3 and VGCCs, respectively, at separate sites. Although the mechanisms that enrich channels at release sites are largely unknown, the RIM-VGCC interaction may provide important molecular insights into the mechanism by which VGCCs are geometrically related to the sites of vesicle fusion.

The question of stoichiometry between VGCCs and vesicle apparatus is intriguing. In this context, Fedchyshyn et al. reported that the coupling of VGCCs to synaptic vesicles at the calyx of Held, a giant excitatory glutamatergic synapse in the auditory brainstem, undergoes a developmental transformation from a "microdomain" to "nanodomain" modality (Fedchyshyn and Wang 2005; Yang et al. 2010). Septin 5 is of critical importance in modulating the spatial proximity between synaptic vesicles and active zones (Yang et al. 2010). In addition to the role of Septin 5, differences in the molecular organization of VGCC complexes may also be implicated. The next challenge in this field is to clarify the molecular organization of presynaptic VGCC complexes in each synapse or at distinct stages of synapse differentiation.

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Chapter 5
Control of Ca_V2 Calcium Channels and Neurosecretion by Heterotrimeric G Protein Coupled Receptors

Mark L. Jewell and Kevin P.M. Currie

Abstract $Ca_V2.1$ (P/Q-type) and $Ca_V2.2$ (N-type) voltage-gated Ca^{2+} channels play pivotal roles in synaptic transmission and neuroendocrine hormone secretion by coupling excitation (i.e. action potential firing) to transmitter release through Ca^{2+}-dependent exocytosis. Consequently, multiple protein-protein interactions and cell signaling pathways converge on these channels to precisely control the amount, location, and timing of Ca^{2+} entry. Among these, G protein coupled receptors (GPCRs) respond to autocrine, paracrine, and retrograde chemical signals to provide important feedback regulation. Several distinct signaling pathways recruited by GPCRs can converge on Ca^{2+} channels, however this chapter focuses on the so-called voltage-dependent inhibition mediated by direct binding of G protein βγ subunits (Gβγ) to the channels. This includes an overview of the functional impact of Gβγ on Ca_V2 channels and current understanding of the molecular mechanisms involved. Neuroendocrine chromaffin cells are also highlighted as both a physiologically important system and powerful cellular model to investigate modulation of Ca^{2+} channels and neurosecretion by GPCRs.

Keywords G protein modulation • GPCRs • Gβγ subunits • Ca_Vβ subunit • Gα subunit • Hormone secretion

5.1 Introduction

Chemical synaptic transmission is fundamental for intercellular communication within the mammalian nervous system, and multipoint regulation is thought to confer synaptic plasticity, and thus complex behavior, learning, and memory.

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Voltage-gated Ca^{2+} channels play pivotal roles in this process by coupling excitation (i.e. action potential firing) to secretion of neurotransmitters through Ca^{2+}-dependent exocytosis. Accordingly, regulation of voltage-gated Ca^{2+} channels, and by extension the intensity/ timing of neurosecretion is critical. Multiple protein-protein interactions and second messenger pathways converge on the channels to control the amount, location, and timing of Ca^{2+}entry, including: direct interaction with the exocytotic fusion machinery including the SNARE proteins syntaxin and SNAP25; phosphorylation by PKC, CaMKII, and other kinases; and complex feedback by Ca^{2+} itself mediated through calmodulin and other related calcium sensing proteins (for recent review see (Catterall and Few 2008)). Another prominent control mechanism, and the subject of this chapter, was first demonstrated ~30 years ago in chick sensory neurons (Dunlap and Fischbach 1978, 1981), and involves inhibition of the Ca^{2+} channels by G protein coupled receptors (GPCRs) (Ikeda and Dunlap 1999; Tedford and Zamponi 2006; Currie 2010a).

Different combinations of pore forming and auxiliary channel subunits (Catterall 2000; Ertel et al. 2000; Yokoyama et al. 2005), all of which are subject to alternate mRNA splicing (Liao et al. 2005; Gray et al. 2007; Flucher and Tuluc 2011; Lieb et al. 2012) or RNA editing (Huang et al. 2012), result in substantial functional diversity of Ca^{2+} channels. Recording from recombinant channels in heterologous expression systems is one powerful tool to investigate ion channel function, and has proven invaluable for assigning specific traits to a particular subunit, and structure-function studies involving mutagenesis. However, it can be challenging to precisely reconstitute all aspects of native Ca^{2+} currents and recording the downstream physiological consequences (i.e. transmitter exocytosis) may not be possible. Recording endogenous channels provides more physiological context, but can be complicated by the presence of multiple channel types, auxiliary subunits, and so on. Typically, neuronal Ca^{2+} currents are recorded from the cell soma, due to the inaccessibility and small size of the presynaptic terminal. For the same reason, in most cases presynaptic transmitter release is monitored indirectly, for example by recording post-synaptic electrical responses or by optical approaches that track labeled synaptic vesicles. Therefore, directly relating channel function to transmitter release can be a challenge, and one also needs to bear in mind that channel modulation might vary in different subcellular compartments (Delmas et al. 2000). There are a few specialized synapses that are amenable to direct electrical recording of presynaptic channels, for example the calyx of Held in the auditory brain stem (Schneggenburger and Forsythe 2006). Moreover, Ca_V2 channels are expressed in chromaffin cells of the adrenal medulla (Garcia et al. 2006; Fox et al. 2008; Currie 2010b), and transmitter release from these small (~10–15 μm), spherical, neuroendocrine cells occurs from the cell soma. This permits direct correlation of GPCR effects on Ca^{2+}channels and transmitter release (Currie 2010b), and provides a powerful model to investigate the cellular/ molecular mechanisms of neurosecretion (Bader et al. 2002; Garcia et al. 2006; Neher 2006).

Below, we review work of many labs investigating the inhibition of "presynaptic" $Ca_V2.1$ and $Ca_V2.2$ channels by GPCRs. Given the scope of the topic, we are unable to include mention of all studies, but rather focus on the Gβγ-mediated mechanism by which GPCRs inhibit neurotransmitter and neuroendocrine hormone release.

5.2 Overview of Voltage-Gated Calcium Channels

In mammals ten genes are known that encode pore forming α1 subunits of voltage-gated Ca^{2+} channels. These are subdivided into three families based on sequence homology: four Ca_V1 members ($Ca_V1.1$– $Ca_V1.4$; all L-type channels), three Ca_V2 members ($Ca_V2.1$, P/Q-type; $Ca_V2.2$, N-type; $Ca_V2.3$, R-type channels) and three Ca_V3 members ($Ca_V3.1$–$Ca_V3.3$, all T-type channels) (Ertel et al. 2000; Catterall et al. 2005). Of these, the $Ca_V2.1$ and $Ca_V2.2$ channels are most closely coupled to neurotransmitter release, and as such are densely localized to brain regions rich in synaptic structures (Westenbroek et al. 1995, 1992; Trimmer and Rhodes 2004). $Ca_V\alpha1$ subunits are large (∼175–225 kDa) proteins consisting of four homologous domains, each domain containing six transmembrane α-helical segments (S1-S6) (Fig. 5.1) (Catterall 2000). The channel adopts a tetrameric architecture, with the pore formed by the S5-S6 and intervening P-loop from each domain, while the S1-S4 segments comprise the voltage sensing regions (Fig. 5.1). The intracellular N- and C-termini and the cytoplasmic loops connecting domains I-IV are all important regulatory regions targeted by a variety of proteins, including the $Ca_V\beta$ subunit, G proteins, SNARE proteins, calmodulin and protein kinases (Fig. 5.1).

The Ca_V1 and Ca_V2 families are high-voltage-activated (HVA) channels that require stronger membrane depolarization to activate relative to the low-voltage-activated Ca_V3 channels. These HVA channels are heteromultimeric complexes that, in addition to the pore forming α1 subunit, contain auxiliary β and $\alpha_2\delta$ subunits (Catterall 2000; Dolphin 2012). The cytoplasmic $Ca_V\beta$ subunit binds to the Alpha Interaction Domain (or AID) on the I-II linker (Fig. 5.1) (Pragnell et al. 1994; Opatowsky et al. 2004; Van Petegem et al. 2004). Four genes are known that encode $Ca_V\beta$ subunits (for reviews see Dolphin 2003; Buraei and Yang 2010). α2δ subunits are the product of a single gene that subsequently undergoes posttranslational cleavage into the α2 and δ portions which are then reconnected by a disulfide bond (Klugbauer et al. 2003). The α2 portion is extracellular while the δ portion is linked to the plasma membrane, potentially through a glycosylphosphatidylinositol (GPI) anchor (Davies et al. 2010). Four genes encode α2δ subunits. Both the β and α2δ subunits control trafficking/ stability and modulate the biophysical properties of the channels (for reviews see Arikkath and Campbell 2003; Bauer et al. 2010; Buraei and Yang 2010; Dolphin 2012). $Ca_V\beta$ also contributes to regulation of the channels by second messengers (Heneghan et al. 2009; Abiria and Colbran 2010; Hermosilla et al. 2011; Suh et al. 2012) and, as detailed below, G proteins (Canti et al. 2000;

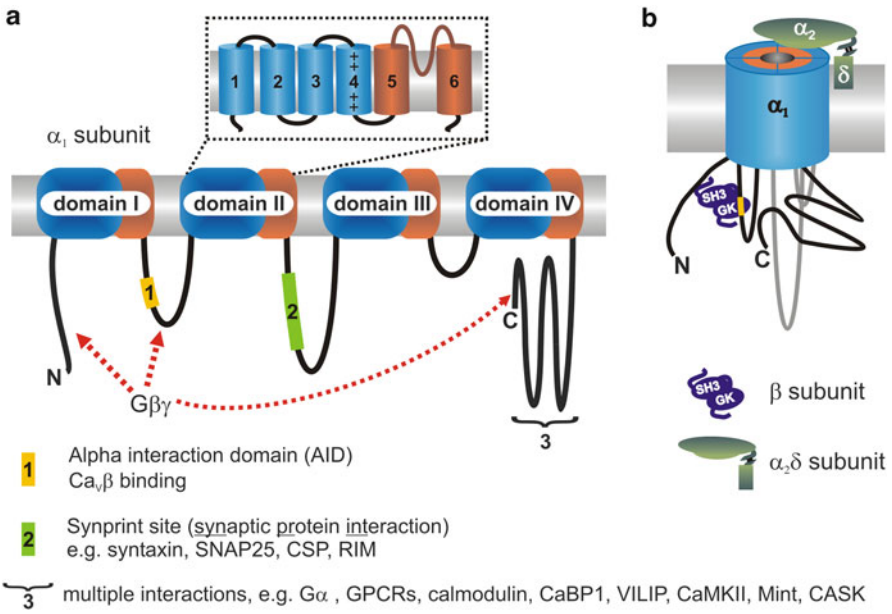

Fig. 5.1 Topology, domain structure and subunit composition of voltage-gated Ca^{2+} channels. (**a**) Topology of the channel α_1 subunit. This pore forming subunit consists of four homologous repeats (domain I through domain IV), which fold to impart an overall tetrameric architecture to the channel. Each domain (see *inset*) has six transmembrane spanning α-helices (S1-S6) (*blue or orange cylinders*). S5, S6 and the P-loop connecting them comprise the 'pore domain' of the channel (*colored orange*), while S1-S4 (in particular S4 that has multiple charged residues) comprises the 'voltage sensor' (*colored blue*). The intracellular N- and C-termini and the cytoplasmic loops that connect domains I–IV are important for interaction with other proteins that modulate channel trafficking, stability, and function, including; the auxiliary β subunit, synaptic proteins, kinases, Gβγ, GPCRs, calmodulin and other Ca^{2+} binding proteins. (**b**) Cartoon depiction of the α1 subunit along with auxiliary β and α2δ subunits. The α1 subunit adopts a tetrameric architecture with the pore forming region of each domain lining the aqueous pore, flanked by the four voltage-sensing domains. The β subunit is cytoplasmic and interacts through its guanylate kinase-like domain (GK) with the I–II linker of the α1 subunit (at the α-interaction domain or AID). The α2δ subunit is largely extracellular and likely GPI-anchored to the plasma membrane

Feng et al. 2001; Leroy et al. 2005; Zhang et al. 2008; Dresviannikov et al. 2009). An additional auxiliary subunit, the γ1 subunit, has also been found to associate with $Ca_V1.1$ channels in skeletal muscle, and several neuronal isoforms including γ2 (also called stargazin) have been identified in neurons (Chen et al. 2007). However, it remains unclear if these proteins constitute *bona fide* Ca^{2+} channel subunits in neurons, where they have been shown to associate with and modulate glutamatergic AMPA receptors (Diaz 2010).

5.3 G Protein Coupled Receptors and Heterotrimeric G Protein Signaling

In addition to targeting ionotropic receptors, many neurotransmitters (e.g. GABA, acetylcholine, glutamate and several neuropeptides) also target cognate GPCRs that can be expressed both pre- and postsynaptically. GPCRs have an extracellular N-terminus of varying size and conformation which includes the agonist binding site (Kristiansen 2004), followed by a characteristic series of seven transmembrane spanning alpha helices and an intracellular C-terminus that couples to heterotrimeric G proteins. At rest, GDP bound G proteins exist as a heterotrimer of α- β- and γ-subunits. Agonist binding to the GPCR causes conformational changes that expose a binding pocket on the intracellular face of the receptor for the Gα C-terminus. In turn, this catalyzes the release of GDP from Gα which is rapidly replaced by GTP. Structural changes in Gα eliminate the Gβγ binding site allowing each liberated subunit (Gα and Gβγ) to initiate downstream signaling cascades (Oldham and Hamm 2008; McIntire 2009). Intrinsic GTPase activity of Gα results in reassociation of Gα-GDP with Gβγ and terminates signaling, a process that is accelerated by RGS (regulator of G protein signaling) proteins (Hollinger and Hepler 2002).

In humans 16 genes encode Gα subunits, five genes encode Gβ, and 12 genes encode Gγ. The heterotrimers, and the receptors to which they couple, are typically classified into one of four families based on sequence homology of the Gα subunit: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, (in addition to $G\alpha_t$ or transducin) (Downes and Gautam 1999; Kristiansen 2004; Oldham and Hamm 2008; Rosenbaum et al. 2009). Gβγ is thought to be an obligate heterodimer, and its crystal structure is visually dominated by the propeller-like folding of Gβ, in which four-stranded β-sheets comprise each of the seven blades of the propeller, and the N-terminus adopts an α-helical domain that interacts with Gγ (Wall et al. 1995) (see Fig. 5.4b). Little is known about how or if different Gβγ dimers confer specificity in targeting downstream effectors.

5.4 GPCRs Can Recruit Several Pathways to Inhibit Ca_V2 Channels

Several distinct signaling pathways recruited by GPCRs can converge on Ca^{2+} channels to inhibit their activity. Broadly speaking, these disparate mechanisms can be classified as either voltage-dependent or voltage-independent. *Voltage-dependent inhibition* is widespread in both the central and peripheral nervous systems and is mediated by a single well defined mechanism involving direct binding of Gβγ to the α1 subunit of Ca_V2 channels (Herlitze et al. 1996; Ikeda 1996). As detailed below, this shifts the voltage-dependence of channel activation, and the inhibition

can be reversed at depolarized membrane potentials. Hence the mechanism is dubbed "*voltage-dependent inhibition*". In contrast, *voltage-independent inhibition* lumps together several other mechanisms that generally develop more slowly and are mediated by a variety of distinct second messenger pathways including phosphorylation, lipid signaling, and channel trafficking (Hille 1994; Elmslie 2003; Michailidis et al. 2007; Roberts-Crowley et al. 2009). The common feature to all these pathways is the inability of strong membrane depolarization to overcome the inhibition. The prominence of voltage-independent inhibition is also more variable, but it seems particularly relevant for controlling somatic Ca^{2+} channels in sensory and sympathetic neurons.

Although there are a few exceptions (Elmslie 1992; Zhu and Ikeda 1994; Currie and Fox 2000), Gβγ-mediated, voltage-dependent inhibition is usually elicited by Gi/o-coupled GPCRs and thus blocked by pertussis toxin. One explanation for this preferential involvement of Gi/o-coupled receptors is co-localization with the channels through adapter proteins like Homer (Kammermeier et al. 2000) or NHERF2 (Filippov et al. 2010). Direct interaction between GPCRs and the channels has also been reported, for example, metabotropic glutamate receptors with $Ca_V2.1$ (Kitano et al. 2003), and dopaminergic (D1/ D2) (Kisilevsky et al. 2008; Kisilevsky and Zamponi 2008) or nociceptin (NOP) receptors (Beedle et al. 2004; Altier et al. 2006) with $Ca_V2.2$. Direct interaction of GPCRs with the channels might also confer additional means of voltage-independent inhibition, such as agonist-mediated endocytosis of the channel/receptor complex (Altier et al. 2006) (but see (Murali et al. 2012)).

Although it is entirely possible that voltage-independent inhibition plays important roles in controlling presynaptic channels, in this chapter we focus on the fast, Gβγ-mediated inhibition thought to underlie rapid modulation of synaptic transmission and neuroendocrine hormone release.

5.5 Characteristic Features of Voltage-Dependent Inhibition: The "Willing-Reluctant" Model

Voltage-dependent inhibition exhibits several characteristic features that provide an identifying biophysical signature (Fig. 5.2): the inhibition is diminished at depolarized membrane potentials; the voltage-dependence of activation is shifted to more depolarized potentials; the activation kinetics are slowed; most of the inhibition is relieved and the channel kinetics are normalized by a conditioning prepulse to depolarized potentials (termed prepulse relief or prepulse facilitation). Voltage-dependent relief of the inhibition can also occur to some extent during more physiologically relevant stimuli such as high frequency trains of action potential-like waveforms (Womack and McCleskey 1995; Brody et al. 1997; Williams et al. 1997; Park and Dunlap 1998; Tosetti et al. 1999; Currie and Fox 2002). In this case the magnitude of facilitation increases with stimulation frequency, and in turn this might contribute to short term synaptic plasticity at some synapses (Brody and Yue 2000).

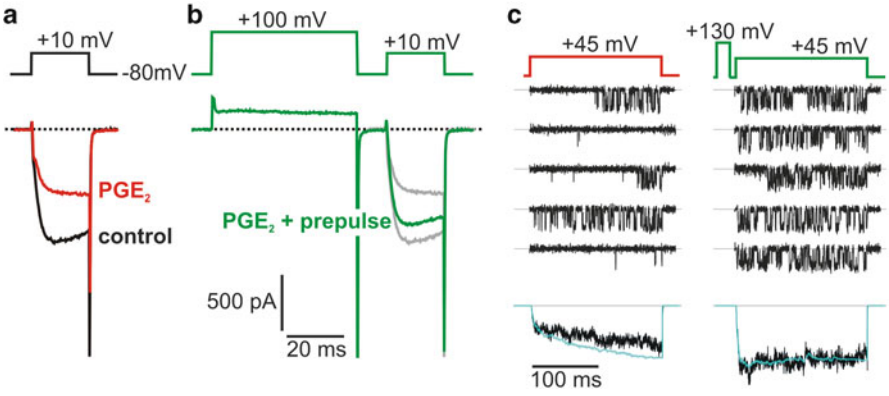

Fig. 5.2 Hallmark features of voltage-dependent inhibition. (**a**, **b**) Example of "whole cell" patch clamp recording of I_{Ca} from an adrenal chromaffin cell. Prostaglandin E_2 (PGE_2) acts through Gi/o-coupled EP3 receptors to inhibit I_{Ca}. The inhibition displays hallmarks of voltage-dependent inhibition: peak amplitude was reduced, activation kinetics were slowed, and in the continued presence of agonist both of these effects were reversed by a conditioning prepulse to + 100 mV (*green trace*) (panel b). (**c**) Example showing voltage-dependent inhibition of single N-type Ca^{2+} channel currents (reproduced with permission of Society for Neuroscience from Colecraft et al. (2001)). Recombinant $Ca_V2.2$ channel currents were recorded in the "on-cell" patch clamp configuration with GPCR agonist included in the patch-pipette to elicit tonic inhibition of the channels in the membrane patch (*left panel*). A conditioning prepulse (to + 130 mV) was used to reverse this inhibition (*right panel*). Five representative current sweeps are shown, along with an ensemble (pseudo macroscopic) current at the bottom of each panel. Inhibited "reluctant" channels display substantially longer latency (time to first channel opening) upon membrane depolarization. The conditioning prepulse normalizes channel gating to that seen under control conditions

As first proposed by Bean (Bean 1989), these characteristic features have been incorporated into models in which the channels exhibit two functional gating states, "willing' and "reluctant" (Bean 1989; Elmslie et al. 1990; Carabelli et al. 1996; Colecraft et al. 2000; Lee and Elmslie 2000). In the absence of Gβγ, the channels predominantly populate the "willing" state, whilst binding of Gβγ favors the "reluctant" state. Voltage-dependent relief of the inhibition, for example by a depolarizing prepulse, is thought to reflect transient dissociation of Gβγ from the channels with a concomitant shift from "reluctant" to "willing" gating states (Fig. 5.3). Although dissociation of Gβγ is not the only mechanism that could underlie facilitation, it is supported by analyses of prepulse relief as a function of agonist or Gβγ concentration. When the concentration of Gβγ was increased, the rate of relief during the prepulse (i.e. dissociation of Gβγ) was not altered. However, the rate of reinhibition following the prepulse was faster, as predicted for rebinding of the Gβγ (Golard and Siegelbaum 1993; Elmslie and Jones 1994; Delmas et al. 1998; Zamponi and Snutch 1998). The concentration-dependence and monoexponential kinetics of reinhibition were also consistent with a bimolecular interaction of a single Gβγ dimer with the channel (Zamponi and

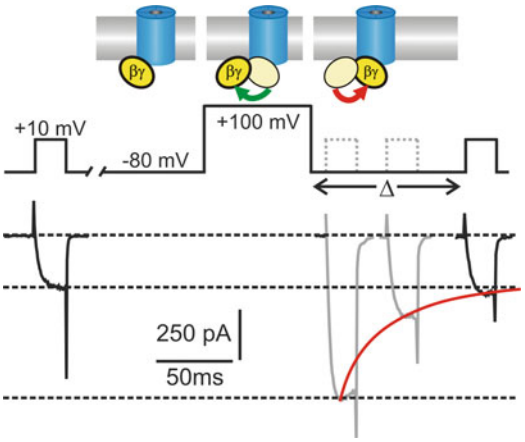

Fig. 5.3 Voltage-dependent relief of inhibition reflects transient dissociation of Gβγ from the channel. The currents shown were recorded from recombinant $Ca_V2.2$ channels expressed with β1b, α2δ in HEK293 cells. Gβγ was co-expressed and produced tonic inhibition of I_{Ca} that was reversed by a conditioning prepulse to + 100 mV. As illustrated by the cartoon, prepulse facilitation is thought to reflect dissociation of Gβγ from an inhibitory binding site on the channel at the depolarized membrane potential. Upon return to the hyperpolarized membrane potential, Gβγ rebinds to, and re-inhibits, the channel. The timecourse of this re-inhibition can be investigated by varying the interval between the prepulse and test pulse (Δ). Re-inhibition of I_{Ca} is well fit with a single exponential (*red line*) and the rate is faster as the local concentration of Gβγ increases

Snutch 1998). Gβγ dissociation also occurs (albeit more slowly) with moderate membrane depolarization, manifest as the slowed activation kinetics of whole cell I_{Ca} (Fig. 5.2a).

5.5.1 Single Channel Investigation of Voltage-Dependent Inhibition

Using the "cell-attached" ("on-cell") recording configuration, several studies showed that agonist must be included in the patch pipette to elicit inhibition (Forscher et al. 1986; Bernheim et al. 1991; Elmslie and Jones 1994). Thus, only GPCRs localized close to the channels (within the membrane patch under the pipette) can couple to and inhibit the channels in that patch. When agonist is bath applied (i.e. outside the patch pipette), the GPCRs on the rest of the cell membrane are activated but do not inhibit the channels, showing that the signaling pathway is "membrane delimited" and does not involve diffusible intracellular messengers. Single channel recordings also reveal the characteristic gating shifts associated with voltage-dependent inhibition (Fig. 5.2c). Upon membrane depolarization, the latency (delay) to first channel opening was increased with little effect on other single channel parameters (Carabelli et al. 1996; Patil et al. 1996). Thus, the

inhibited ("reluctant") channels appear essentially silenced, unable to open until Gβγ dissociated and the channels shift to the "willing" state. Subsequently it has been reported that $Ca_V2.2$ but not $Ca_V2.1$ channels can display very brief channel openings from the "reluctant" state (i.e. without Gβγ unbinding), although the probability of such events was low (Colecraft et al. 2000; Lee and Elmslie 2000).

5.5.2 *Alteration of Gating Currents by Gβγ*

Gating currents of voltage-gated channels are not due to ionic flux through the channel pore, but rather reflect movement of the charged voltage-sensor domain of the channels in response to membrane potential changes. Expression of recombinant $Ca_V2.2$ in HEK293 cells enables recording of these gating currents in isolation as the cells lack other endogenous voltage-gated channels. Using this approach it was found that Gβγ reduced the amplitude, and shifted the voltage-dependence of gating currents to more depolarized potentials (Jones and Elmslie 1997), again consistent with the channels entering a "reluctant" state. G proteins also produced a significant separation in the voltage-dependent activation of gating current and ionic current (Jones and Elmslie 1997). Together these data suggest that Gβγ binding slows movement of the voltage-sensor and uncouples this movement from opening of the channels. Similar modulation of gating currents by G proteins has also been reported in rat sympathetic neurons (Hernandez-Ochoa et al. 2007; Rebolledo-Antunez et al. 2009).

5.5.3 *Gβγ Modulates Channel Inactivation*

In addition to these dominant effects on channel activation, evidence shows that Gβγ can also modulate *inactivation* of $Ca_V2.2$ channels (McDavid and Currie 2006; Weiss et al. 2007). Although the precise molecular correlates remain somewhat unclear, fast voltage-dependent inactivation might involve a "hinged lid" mechanism in which the pore is occluded by the intracellular loop connecting domains I and II of the α_1-subunit (Stotz and Zamponi 2001; Tadross et al. 2010) (but see Findeisen and Minor 2009). The I-II linker is also important for binding Gβγ (Herlitze et al. 1997; De Waard et al. 1997, 2005; Schiff et al. 2000) (Fig. 5.1) (see below for more discussion), so it is feasible that this could disrupt movement or interaction of this putative inactivation gate with other channel domains. Ca_V2 channels can also inactivate from intermediate closed state(s) favored during trains of brief repetitive stimuli (Patil et al. 1996). Gβγ could also reduce the cumulative inactivation throughout a stimulus train by reducing the probability that the channels populate the state from which inactivation is preferred. In addition to voltage-dependent mechanisms, the channels can also undergo Ca^{2+}-dependent inactivation mediated through calmodulin interaction with the C-terminus of the channel (Lee et al. 1999, 2003;

Peterson et al. 1999; Zuhlke et al. 1999; Liang et al. 2003). The reduction of Ca^{2+}-dependent inactivation by Gβγ (McDavid and Currie 2006) might therefore result from fewer channels opening and a diminished "global" Ca^{2+} signal, or through more complex interactions perhaps including binding of Ca^{2+}-calmodulin to Gβγ which has been reported to occur at least in vitro (Liu et al. 1997).

5.6 $Ca_V2.2$ Channels Are More Susceptible to Gβγ-Mediated Inhibition than $Ca_V2.1$ Channels

Ca^{2+} entry through $Ca_V2.1$ and/or $Ca_V2.2$ channels triggers neurotransmitter release at most synapses, and both of these channels are inhibited by Gβγ. However, the magnitude of inhibition is greater for N-type ($Ca_V2.2$) than for P/Q-type ($Ca_V2.1$) I_{Ca} (Bourinet et al. 1996; Zhang et al. 1996; Currie and Fox 1997). Reversal of $Ca_V2.2$ inhibition during high frequency bursts of action potentials occurs to a lesser extent than for $Ca_V2.1$ and is more sensitive to changes in the action potential amplitude and duration (Currie and Fox 2002). These differences are consistent with higher affinity binding of Gβγ to $Ca_V2.2$. Indeed, although the apparent affinity of Gβγ for the two channel types is similar at hyperpolarized or very depolarized potentials, there is a significant divergence at moderately depolarized potentials ($<+30$ mV) (Colecraft et al. 2000). These data all suggest that GPCR mediated inhibition of neurotransmission would be more effective at synapses expressing $Ca_V2.2$ compared to those expressing $Ca_V2.1$ channels. Furthermore, although changes in the relative contribution of the two channel types might have little effect on transmitter release per se, it could significantly change neuromodulation by GPCRs (Brody and Yue 2000; Cao and Tsien 2005; Inchauspe et al. 2007).

5.7 Regions of the Calcium Channel that Mediate Inhibition by Gβγ

Cumulative evidence from a variety of approaches (mutagesis, chimeric channels, peptide mimetics/blockers) suggests that multiple sites on the α1 subunit of the channel comprise a binding pocket for Gβγ. Two distinct binding sites for Gβγ have been reported on the I-II linker (De Waard et al. 1997; Herlitze et al. 1997; Zamponi et al. 1997; Tedford et al. 2010). The first site has a consensus sequence for Gβγ binding found in phospholipase C β2 and type 2 adenylyl cyclase (QXXER). This site (containing QQIER in all three Ca_V2 channel members) overlaps with the binding site for the Ca_Vβ subunit (the AID) (Pragnell et al. 1994; Van Petegem et al. 2004). A second site further along the I-II linker has also been identified (De Waard et al. 2005). In vitro binding assays between Gβγ and the I-II linker peptide

show high affinity interactions (20–60 nM) (De Waard et al. 1997; Zamponi et al. 1997; Bell et al. 2001), although this is reduced somewhat by the presence of a $Ca_V\beta$ subunit (Zhang et al. 2008). Peptides based on both sites diminish voltage-dependent inhibition of the channels and point mutations introduced into the sites can either reduce or enhance inhibition (Tedford et al. 2010). PKC can reduce voltage-dependent inhibition of $Ca_V2.2$ (N-type) I_{Ca} (Swartz 1993; Zamponi et al. 1997; Barrett and Rittenhouse 2000; Simen et al. 2001; Bertaso et al. 2003), and this has been linked to phosphorylation of Thr^{422} on the I-II linker (of the rat $Ca_V2.2$), close to the second $G\beta\gamma$ binding site (Zamponi et al. 1997; Hamid et al. 1999). Of note, phosphorylation of Thr^{422} disrupts the inhibition of I_{Ca} mediated by $G\beta_1$, but not other $G\beta$ subunits (Cooper et al. 2000), and two residues on $G\beta1$ (Asn^{35} and Asn^{36}) have been shown to underlie this difference (Doering et al. 2004). These data also suggest that Thr^{422} on the rat $Ca_V2.2$ I-II linker and $G\beta\gamma$ come into close proximity with one another during inhibition. Evidence implicating the I-II linker has been less clear in some other studies. For example, chimeric channels in which the I-II linker of $Ca_V1.2$ was introduced into the $Ca_V2.2$ backbone were still inhibited (Zhang et al. 1996; Canti et al. 1999; Agler et al. 2005).

The N-terminus has also been identified as crucial for voltage-dependent inhibition (Simen and Miller 1998; Stephens et al. 1998; Canti et al. 1999; Agler et al. 2005). Evidence for this emerged from the finding that a short splice variant of $Ca_V2.3$ channels with a truncated N-terminus was not inhibited by $G\beta\gamma$, whereas a splice variant with an intact N-terminus was (Page et al. 1998). The Dolphin lab further demonstrated that truncating the N-terminal 55 amino acids of $Ca_V2.2$ prevented voltage-dependent inhibition, whereas introducing the $Ca_V2.2$ N-terminus into the $Ca_V1.2$ backbone conferred modest inhibition onto these normally resistant channels (Page et al. 1998; Canti et al. 1999). An 11 amino acid stretch of the N-terminus (residues 45–55) predicted to form an α-helix (Page et al. 2010) seems critical for $G\beta\gamma$-mediated inhibition, especially residues S48, R52 and R54, with I49 involved to a lesser extent (Canti et al. 1999). The Yue lab demonstrated that $G\beta\gamma$ interacts directly with the N-terminus and also showed that the N-terminus (residues 56–95) directly binds to the I-II linker from $Ca_V2.2$ but not $Ca_V1.2$ (Agler et al. 2005). Thus, the N-terminus may contribute to a binding pocket for $G\beta\gamma$ and, through intra-molecular interaction with the I-II linker, serve as an "inhibitory module" that mediates the functional shift from willing to reluctant gating states. A recent study reported that peptides based on the N-terminus (residues 45–55 of rat $Ca_V2.2$) or AID of the channel (377–393 of rat $Ca_V2.2$) suppressed I_{Ca} and transmitter release from superior cervical ganglion neurons, and diminished $G\beta\gamma$-mediated inhibition (Bucci et al. 2011). It was concluded that peptide interaction with the channels partially recapitulated and occluded the shifts in channel gating produced by $G\beta\gamma$.

The C-terminus of the channel has also been reported to play an important role in modulation of $Ca_V2.3$ (Qin et al. 1997). However, large parts of the C-terminus can be deleted in $Ca_V2.2$ channels with little impact on the extent of voltage-dependent inhibition (Furukawa et al. 1998; Hamid et al. 1999). Thus, it might play a modulatory role in $Ca_V2.2$ channel regulation, perhaps by increasing the affinity

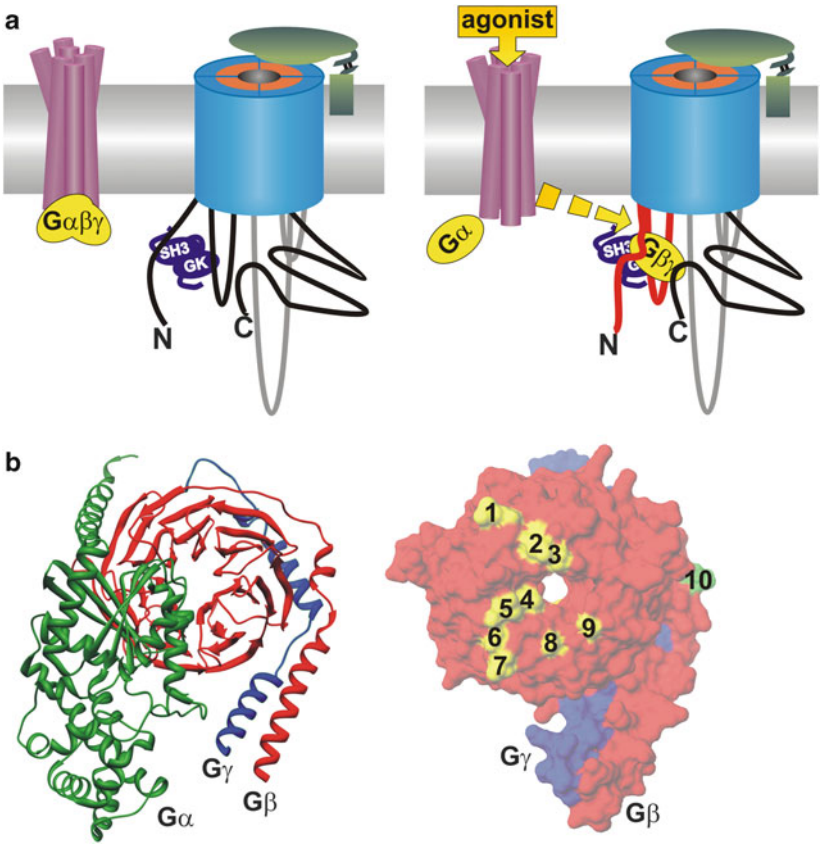

Fig. 5.4 Cartoon model depicting the molecular interactions that underlie Gβγ-mediated inhibition of Ca_V2 channels. (**a**) Depicts a channel, GPCR, and heterotrimeric G protein under basal conditions (no agonist; *left panel*). Upon GPCR stimulation (*right panel*), Gβγ dissociates and is free to interact with effector proteins including Ca_V2 channels. Mutagenesis and other approaches suggest the Gβγ binding pocket is comprised from multiple sites on the N-terminus, I-II linker, and probably C-terminus of the channel. Gβγ binding promotes interaction of the N-terminus "inhibitory module" with the initial one-third of the I-II-linker. This (and perhaps other interactions) shifts the channels to reluctant gating states and results in functional inhibition. Although not required for inhibition per se, binding of a $Ca_V\beta$ subunit to the AID on the I-II linker is necessary for voltage-dependent reversal of the inhibition by strong depolarizations. (**b**) *Left panel*: Ribbon diagram showing the structure of a heterotrimeric G protein ($G\alpha_i$ – *green*; $G\beta_1$ – *red*; and $G\gamma_2$ *blue*). Many effectors bind to a protein interaction "hot spot" on the surface of Gβ that is masked by Gα in the heterotrimer. Activation by a GPCR results in dissociation of Gα and unmasking of this effector interaction face of Gβγ. *Right panel*: Molecular surface rendering of the Gα interacting face of Gβγ. Mutagenesis of the residues marked in *yellow* has been reported to disrupt inhibition of Ca_V2 channels. (1 = L55; 2 = K57; 3 = W332; 4 = M101; 5 = L117; 6 = M119; 7 = T143; 8 = D186; 9 = D228). Residues marked in *green* (10 = N35, N36) are involved in crosstalk between Gβ1 and PKC phosphorylation of $Ca_V2.2$. Images were generated using the UCSF Chimera package (Pettersen et al. 2004; Sanner et al. 1996) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco using data reported by Wall et al. (1995) (PDB ID: 1GP2)

of Gβγ binding (Li et al. 2004). Of note, the C-terminus does bind a number of other proteins including calmodulin, CaMKII, PKC, and Gα subunits (Evans and Zamponi 2006; Catterall and Few 2008), which could facilitate crosstalk between Gβγ-mediated inhibition and other signaling pathways (Simen et al. 2001; Bertaso et al. 2003).

To summarize, it appears that the binding site for Gβγ is comprised from multiple sites on the N-terminus, I-II linker, and perhaps the C-terminus of the channel. Upon binding of Gβγ, the N-terminus (residues 56–95) interacts with the proximal one-third of the I-II-linker (see Fig. 5.4a). This (and perhaps other interactions) presumably underlies the shift in channel gating from "willing" to "reluctant".

5.8 Regions of Gβγ Implicated in the Inhibition of Ca_V2 Channels

Several studies have also used mutagenesis approaches to identify residues on Gβγ involved in inhibiting Ca_V2 channels. High resolution crystal structures are available showing that Gβ exhibits a seven blade β-propeller structure with an α-helical N-terminus that binds Gγ (Wall et al. 1995; Gaudet et al. 1996; Lambright et al. 1996; Sondek et al. 1996; Lodowski et al. 2003) (see Fig. 5.4b). In the heterotrimer, binding of Gα to Gβ masks a protein interaction "hot spot" that contains overlapping subsets of residues involved in many effector protein interactions (Fig. 5.4b) (Smrcka 2008). Most mutagenesis studies that disrupt inhibition of Ca_V2 channels have identified residues on this Gα interacting surface (Ford et al. 1998; Agler et al. 2005; McDavid and Currie 2006; Tedford et al. 2006) (Fig. 5.4b). Three residues on the opposite face of $Gβ_1$ have also been implicated (Mirshahi et al. 2002; Doering et al. 2004; Tedford et al. 2006), while two (Asn^{35} and Asn^{36}) appear to underlie the ability of PKC to antagonize inhibition of $Ca_V2.2$ by $Gβ_1$ (Doering et al. 2004). Less is known about the role of Gγ, although it has been reported that different isoforms of Gγ can influence the extent of inhibition (Zhou et al. 2000; Blake et al. 2001).

5.9 Influence of the Calcium Channel β Subunit on Gβγ-Mediated Inhibition

$Ca_Vβ$ subunits exert a variety of effects on Ca^{2+} channels from trafficking, modulation of channel kinetics, and recruitment of signaling complexes (for reviews see (Buraei and Yang 2010; Dolphin 2012). It has also been shown that the magnitude and kinetics of voltage-dependent inhibition depends on the subtype of $Ca_Vβ$ and G protein β subunit involved (Canti et al. 2000; Feng et al. 2001). Recent evidence from the Dolphin and Yang labs show that binding of $Ca_Vβ$ to the $Ca_Vα1$ subunit

is not required for Gβγ-mediated inhibition per se, but is required for voltage-dependent reversal of that inhibition (Meir et al. 2000; Leroy et al. 2005; Zhang et al. 2008; Dresviannikov et al. 2009).

The Dolphin lab introduced a mutation (W391A) into the AID on the I-II linker of $Ca_V2.2$ channels which reduces $Ca_V\beta$ subunit binding affinity by ~1,000 fold. (Leroy et al. 2005). Altered gating kinetics and reduced current density (due to disrupted trafficking) confirmed the channels lacked a $Ca_V\beta$ subunit. The magnitude of inhibition evoked by D2 dopamine receptors or exogenous Gβγ was similar to that seen in wild-type channels, however prepulse reversal of the inhibition was lost in the W391A mutant. Mutation of an additional two residues shown to be essential for Gβγ-mediated inhibition (R52A and R54A on the N-terminus) abolished this voltage-independent inhibition in W391A channels. When the β_{2a} subunit was expressed with the W391A channels rather than the β_{1b} subunit, voltage-dependent relief of the Gβγ mediated inhibition was restored. This was attributed to palmitoylation of the β_{2a} subunit at two N-terminal cysteine residues, because when these were mutated voltage-dependent relief was lost (i.e. the data resembled β_{1b}). The authors proposed that palmitoylation effectively increases the local plasma membrane concentration of β_{2a} and thereby promotes low affinity interaction with the α_1 subunit. A follow up study demonstrated essentially the same findings, intact inhibition but loss of voltage-dependent reversal, in cells transfected with wild type $Ca_V2.2$ and $\alpha_2\delta$ (but without $Ca_V\beta$) (Dresviannikov et al. 2009).

The Yang lab investigated inhibition of $Ca_V2.1$ channels and chose to mutate $Ca_V\beta$ to reduce the affinity for the AID (Zhang et al. 2008). The channels were expressed in Xenopus oocytes and macroscopic currents recorded from giant inside-out patches. Due to the reduced binding affinity of the mutant $Ca_V\beta$ subunit, washing the cytoplasmic face of the patches resulted in loss of binding which was confirmed by the expected shifts in channel kinetics compared to wild type. These channels lacking $Ca_V\beta$ were still inhibited by application of Gβγ, but prepulse reversal of this inhibition was abolished.

The $Ca_V\beta$ subunit consists of SH3 and GK domains separated by a variable HOOK region (Dolphin 2003; Buraei and Yang 2010). Expression of the isolated GK domain (which binds the AID) was sufficient to confer voltage-dependent reversal of Gβγ-mediated inhibition (Zhang et al. 2008; Dresviannikov et al. 2009). It has been reported that the AID adopts a random coil and that binding of $Ca_V\beta$ induces an α-helical conformation that extends back to the interface with domain I (Chen et al. 2004; Opatowsky et al. 2004; Van Petegem et al. 2004; Arias et al. 2005). Disruption of this α-helical structure by introducing seven glycines between the AID and IS6 did not prevent inhibition by Gβγ, but did prevent voltage-dependent reversal in the presence of $Ca_V\beta$ (Zhang et al. 2008).

Overall, it appears that Gβγ binds to a pocket formed by the N-terminus, I-II linker and perhaps C-terminus of the channel. In doing so, it promotes interaction of the channel N-terminus and I-II-linker, disrupts voltage-sensor movement and coupling to channel activation, thereby shifting the channels from "willing" to "reluctant". With strong depolarization, a rigid α-helix produced by binding of $Ca_V\beta$

to the AID might relay movement of the voltage-sensor/ activation gate downstream to the I-II linker to alter the binding pocket, resulting in unbinding of Gβγ and reversal of the inhibition.

5.10 Influence of SNAREs and Other Synaptic Proteins on Gβγ-Mediated Inhibition

The SNARE proteins syntaxin 1A and SNAP25 can bind directly to Ca_V2 channels via the synaptic protein interaction (synprint) site on the domain II-III linker of the channel (Fig. 5.1) (Sheng et al. 1994, 1997; Bezprozvanny et al. 1995; Rettig et al. 1996; Wiser et al. 1997; Zhong et al. 1999). This has several consequences, such as helping to ensure efficient stimulus-secretion coupling by targeting the channels near to vesicle release sites (Mochida et al. 1996). Binding of syntaxin-1 results in a hyperpolarizing shift in voltage dependent inactivation of Ca_V2 channels, which is reversed with the further addition of SNAP -25 (Bezprozvanny et al. 1995; Bergsman and Tsien 2000; Jarvis and Zamponi 2001; Davies et al. 2011) (for review see (Davies and Zamponi 2008)). This might serve to effectively shunt extracellular Ca^{2+} entry through Ca_V2 channels associated with a docked vesicle/t-SNARE complex, and inhibit Ca^{2+} entry through incomplete signaling complexes. Gβγ also binds syntaxin 1, but at a site distinct from that for Ca_V2 channels (Jarvis et al. 2002; Davies et al. 2011). This promotes tonic inhibition, presumably by colocalizing Gβγ and $Ca_V2.2$ channels (Jarvis et al. 2000, 2002; Davies et al. 2011). In contrast, even though syntaxin 1B binds both Gβγ and the channel, it does not promote tonic inhibition (Lu et al. 2001), perhaps suggesting a difference in the spatial orientation of the syntaxin/Gβγ complex relative to the channel. Botulinum neurotoxin C, which cleaves syntaxin, diminished inhibition of Ca^{2+} channels in neuronal preparations supporting the notion that this interaction is physiologically important (Stanley and Mirotznik 1997; Silinsky 2005). Cysteine string protein (CSP) also interacts with G proteins and the synprint site to promote Gβγ-mediated inhibition (Magga et al. 2000; Miller et al. 2003). Evidence is also mounting that Gβγ binding to the SNARE proteins (syntaxin and SNAP25) has direct inhibitory effects on neurosecretion in addition to those mediated through presynaptic Ca^{2+} channels (Blackmer et al. 2001; Gerachshenko et al. 2005; Yoon et al. 2008, 2007) (for reviews see Stephens 2009; Betke et al. 2012).

Interaction with other synaptic proteins might diminish rather than enhance Gβγ-mediated inhibition of Ca^{2+} channels. For example, RIMs (rab3 interacting molecules) have emerged as important organizers of the presynaptic active zone (Sudhof 2012), and can bind Ca^{2+} channels directly, or through interaction with RIM binding proteins or the $Ca_V\beta$ subunit (Hibino et al. 2002; Kiyonaka et al. 2007; Uriu et al. 2010; Han et al. 2011; Kaeser et al. 2011; Gandini and Felix 2012). Coexpression of Rim1 with $Ca_V2.2$ in HEK293 cells has complex effects and promotes "deinhibition" (recovery from inhibition during depolarization) perhaps in part through dramatic slowing of channel inactivation (Weiss et al. 2011). It has

also been reported that stargazin (aka the Ca^{2+} channel γ2 subunit), although not covalently bound to the channel complex, scavenges Gβγ in Xenopus oocytes to reduce inhibition of $Ca_V2.2$ channels (Tselnicker et al. 2010). And, as already noted (section 5.7), PKC can reduce voltage-dependent inhibition of $Ca_V2.2$ channels (Swartz 1993; Zamponi et al. 1997; Barrett and Rittenhouse 2000; Simen et al. 2001; Bertaso et al. 2003), likely through phosphorylation of the channel I-II linker (Zamponi et al. 1997; Hamid et al. 1999), or perhaps in some cases through phosphorylation of the GPCR (Wu et al. 2002).

5.11 Control of Neuroendocrine Hormone Secretion by Gβγ-Mediated Inhibition of Ca_V2 Channels

As already noted, Gβγ-mediated inhibition of Ca_V2 channels is thought to underlie rapid presynaptic inhibition of neurotransmitter release, as recently reviewed elsewhere (Stephens 2009). Here, we briefly outline how neuroendocrine cells can provide experimental advantages and mechanistic insight into the control of neurosecretion by GPCRs, with a focus on catecholamine release from adrenal chromaffin cells.

Chromaffin cells are derived from the neural crest (Huber et al. 2009), and act essentially as postganglionic sympathetic neurons but, rather than innervating a specific postsynaptic target, release catecholamines and a variety of other neuropeptides and hormones into the bloodstream. These transmitters then exert powerful control over the cardiovascular, endocrine, immune, and nervous systems, for example coordinating the "fight-or-flight" response to acute stress. In addition to their physiological importance, chromaffin cells confer significant experimental advantages. The small (∼10–15 μm), spherical cells are well suited for patch clamp electrophysiology to not only record ion channel currents, but also membrane capacitance which precisely reflects the surface area of the cell so can track exocytosis and endocytosis with millisecond time resolution (Gillis 2000; Borges et al. 2008; Yao et al. 2012) (Fig. 5.5). Direct electrochemical monitoring of catecholamine release is also possible using carbon fiber amperometry (Wightman et al. 1991; Travis and Wightman 1998; Borges et al. 2008). With suitable stimulation protocols transient amperometric current "spikes" can be resolved, each of which can be analyzed to determine the amount and kinetics of catecholamine release from individual vesicular fusion events (Mosharov and Sulzer 2005; Machado et al. 2008) (Fig. 5.5). These approaches can also be combined with other techniques including electron microscopy, fluorescent imaging, and photorelease of "caged" Ca^{2+}. The ability to deliver precisely controlled stimuli, and simultaneously record ion channel activity and exocytosis/ transmitter release from the same cellular compartment enables direct cause-and-effect assessment of mechanisms that control neurosecretion. It also enables dissection of the various steps in the exocytotic process, and how those are altered in response to neuromodulators. Of course, there are differences between

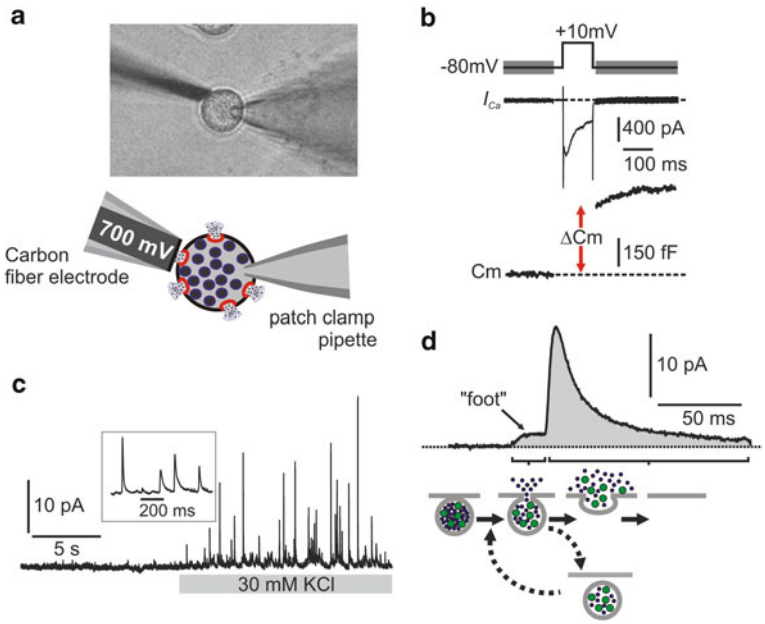

Fig. 5.5 Adrenal chromaffin cells are well suited for investigating stimulus-secretion coupling at the cellular level. (**a**) Photograph and cartoon depiction of a single chromaffin cell with a patch clamp pipette and carbon fiber amperometry electrode in position. (**b**) Vesicle fusion (exocytosis) and recycling (endocytosis) can be tracked as changes in membrane capacitance (Cm) using patch clamp electrophysiology. The upper trace represent the voltage-command applied to the cell, including a sine wave (*grey box*) superimposed on the holding potential. The step depolarization evoked an inward Ca^{2+} current (I_{Ca}, *middle trace*), which in turn evoked vesicular exocytosis detected as a jump in membrane capacitance (ΔCm, *bottom trace*). (**c**) Direct electrochemical detection of catecholamine exocytosis by carbon fiber amperometry. The example shows the amperometric current from the carbon fiber electrode elicited from a non-voltage-clamped chromaffin cell by 30 mM KCl. Each upward "spike" is due to catecholamine release from a single vesicular fusion event. The inset shows a few spikes on an extended time scale. (**d**) An amperometric spike due to oxidation of catecholamines released during a single vesicular fusion event is shown. The charge of the spike (integral – *grey shading*) is directly proportional to the number of catecholamine molecules released. Other kinetic features of the spike can also be analyzed. Some spikes (~1 in 3) display a smaller amplitude plateau or pre-spike "foot" that is thought to reflect release of catecholamine through a narrow fusion pore. As illustrated in the cartoon, the fusion pore may then expand irreversibly resulting in full collapse of the vesicle into the plasma membrane (*solid arrows*), or may open transiently resulting in partial emptying of the vesicular content and rapid recycling of the vesicle

chromaffin cells and neurons (as is also the case between different types of neurons) (Neher 2006). For example, catecholamines are stored and released from large dense core granules rather than small synaptic like vesicles. Nonetheless, chromaffin cells provide both a physiologically important system and powerful cellular model to investigate neurosecretion and its modulation by GPCRs (Currie 2010b).

In the intact gland, chromaffin cells are innervated by splanchnic nerve terminals that release acetylcholine (ACh) and neuropeptide cotransmitters such as PACAP. This sympathetic drive depolarizes the chromaffin cells promoting Ca^{2+} influx through voltage-gated Ca^{2+} channels which triggers fusion of the vesicles with the plasma membrane (Douglas 1968; Boarder et al. 1987), and also modulates other steps such as vesicle trafficking, docking, priming, and recycling via endocytosis (Smith et al. 1998; Chan et al. 2003; Neher and Sakaba 2008; Pasche et al. 2012; Yao et al. 2012). Consequently, as at presynaptic terminals, Ca^{2+} channels play a pivotal role in stimulus-secretion coupling and are an important target for regulation by GPCRs. Chromaffin cells express several subtypes of Ca^{2+} channel, with $Ca_V2.1$ and $Ca_V2.2$ channels accounting for ~50–90 % of the whole cell Ca^{2+} current (depending on species) along with members of the Ca_V1 family (Garcia et al. 2006; Fox et al. 2008; Marcantoni et al. 2008). Functional Ca_V3 channels are not usually seen except in cells from neonates, but can be recruited by hypoxia, chronic β-adrenergic receptor stimulation, or acute application of the stress mediator PACAP (Novara et al. 2004; Carabelli et al. 2007; Marcantoni et al. 2008; Souvannakitti et al. 2010; Hill et al. 2011). The cells also express multiple types of GPCR that sense and respond to changes in local autocrine/ paracrine signals as well as the overall physiological "status" of the animal through neuronal input and endocrine hormones. In general, GPCRs that couple to G_i-type G proteins inhibit catecholamine release, whereas Gq-coupled and Gs-coupled receptors potentiate catecholamine release (Currie 2010b).

Robust voltage-dependent inhibition of Ca_V2 channels is seen in chromaffin cells and contributes to autocrine/ paracrine regulation of catecholamine release (Fig. 5.6). By carefully controlling plating density, and bath perfusion conditions endogenous ATP released from chromaffin cells was shown to mediate autocrine inhibition of I_{Ca} (Currie and Fox 1996). The ATP (and ADP) released from the cells acts on P2Y purinergic receptors, and autocrine μ-opioid receptors that respond to endogenous opiates also play a similar role (Albillos et al. 1996; Currie and Fox 1996). This autocrine inhibition was also demonstrated at the single channel level, in which transmitters released into the cell attached recording pipette inhibited the channels in that patch of membrane (Carabelli et al. 1998). Prostaglandins and perhaps other inflammatory mediators released from macrophages also potently inhibit $I_{Ca,}$ and might underlie local paracrine crosstalk between the immune and neuroendocrine cells types (Currie et al. 2000; Currie and Fox 2000; Jewell et al. 2011). Chromaffin cells have also proven useful for dissecting the differential inhibition of $Ca_V2.1$ and $Ca_V2.2$ channels by Gβγ in a native neurosecretory environment (Currie and Fox 1997). This was achieved by a combination of P2Y autoreceptor activation before and after selective block of different channel types with peptide toxins.

As already noted, one advantage of chromaffin cells is the ability to directly correlate channel inhibition with transmitter release detected by changes in membrane capacitance or amperometry. For example, inhibition of I_{Ca} by P2Y purinergic receptors or prostaglandin EP3 receptors resulted in a parallel inhibition of exocytosis in the same cells (Fig. 5.6) (Harkins and Fox 2000; Powell et al. 2000; Ulate et al.

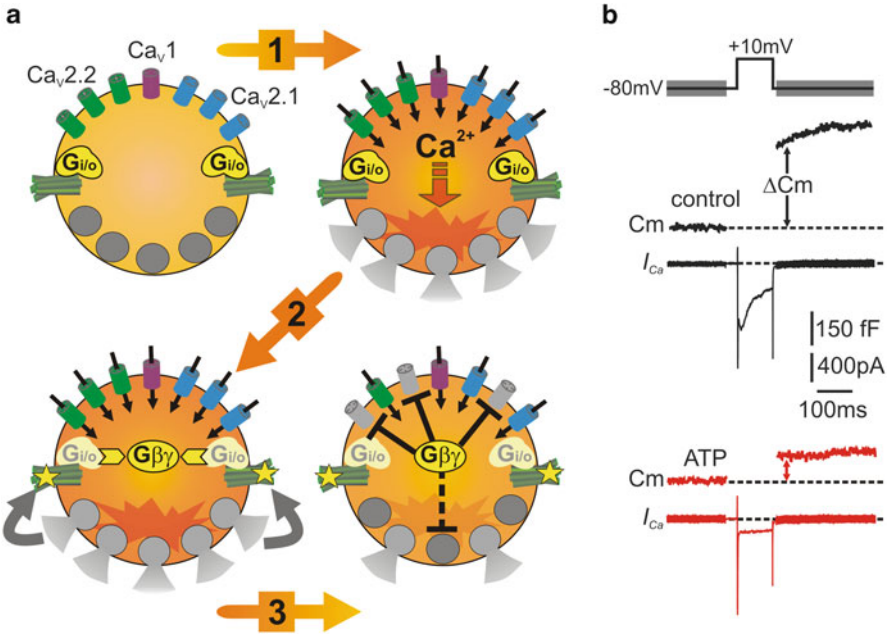

Fig. 5.6 GPCR mediated autocrine inhibition of I_{Ca} and exocytosis in chromaffin cells. (**a**) Cartoon represents a single cell throughout the course of stimulation. At resting membrane potentials, intracellular Ca^{2+} is low. (1) Upon depolarization, voltage-gated Ca^{2+} channels open, leading to Ca^{2+} influx which in turn triggers exocytotic release of catecholamines and other vesicular content. (2) As at presynaptic terminals, autocrine activation of Gi/o-coupled GPCRs, most notably for ATP/ADP (P2Y receptors) and endogenous opiates (μ-opioid receptors), liberates Gβγ. (3) The free Gβγ dimers bind to Ca_V2 channels, inhibiting Ca^{2+} entry though $Ca_V2.2$ to a greater extent than through $Ca_V2.1$. In turn the reduced Ca^{2+} entry results in reduced exocytosis. Gβγ might also inhibit secretion by an additional mechanism(s), perhaps through direct interaction with the exocytotic machinery. (**b**) Example showing inhibition of Ca^{2+} current and exocytosis (change in membrane capacitance) by P2Y autoreceptors. The upper trace represents the voltage-command applied to the cell (*grey box* represents 1 kHz sine wave to monitor Cm), the middle trace shows the change in membrane capacitance (ΔCm) that reflects vesicle fusion (exocytosis), and the lower trace shows the inward Ca^{2+} channel current (I_{Ca}). Activation of P2Y autoreceptors by extracellular ATP (*red traces*) reduced I_{Ca} and exocytosis (ΔCm) in parallel

2000; Jewell et al. 2011). When the inhibition of I_{Ca} was reversed by a depolarizing prepulse (as already discussed above – Fig. 5.2) the inhibition of exocytosis was also diminished. Also, when exocytosis was triggered by intracellular photorelease of "caged Ca^{2+}" to bypass the plasma membrane Ca^{2+} channels, the inhibition of exocytosis was no longer evident (Powell et al. 2000). These data suggest that under conditions with brief intense stimuli, inhibition of I_{Ca} is the dominant mechanism that inhibits exocytosis. That being said, another study proposed both Ca^{2+} channel-dependent and independent inhibition of exocytosis by P2Y receptors (Lim et al. 1997). More recently, Gβγ was reported to reduce both the number and charge of individual amperometric spikes (directly proportional to the number

of catecholamine molecules released by each vesicular fusion event), independent from effects on Ca^{2+} channels. Ca^{2+} channel independent inhibition of synaptic neurotransmitter release has also been reported and evidence points to interaction of Gβγ with the SNARE proteins syntaxin and SNAP25 as a potential mechanism (for recent review see Betke et al. 2012). Chromaffin cells will provide a useful model for dissecting this novel mechanism, determining its physiological relevance, and how it interacts with inhibition of Ca^{2+} channels to control exocytosis under different stimulation paradigms.

5.12 Concluding Remarks

In this review we have highlighted the complex inhibition of Ca_V2 channels by G protein coupled receptors. Voltage-dependent inhibition, mediated by direct binding of Gβγ to the Ca^{2+} channel α1 subunit, is the most common and best understood mechanism. Membrane potential, firing patterns, channel subunit composition/ splice variants, and Gβγ heterodimer composition all modulate the extent and/or kinetics of voltage-dependent inhibition. Although less well understood and perhaps less widespread, there are also several mechanisms leading to voltage-independent inhibition of Ca_V2 channels. These include direct interaction with GPCRs, inhibition through lipid signaling pathways, and channel phosphorylation. Ca_V2 channels are also subject to a variety of other regulatory mechanisms, notably Ca^{2+}-dependent feedback (both inactivation and facilitation). Thus, GPCRs in combination with Ca^{2+} channels sense and integrate a complex array of inputs in order to fine tune the spatiotemporal aspects of Ca^{2+} entry that play such pivotal roles in cellular physiology, synaptic transmission, and neuroendocrine hormone release.

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Chapter 6
RGK Small GTPases and Regulation of Ca_V2 Channels

Pierre Charnet, Frédérique Scamps, Matthieu Rousset, Claudine Menard, Michel Bellis, and Thierry Cens

Abstract About 10 years ago, a yeast two-hybrid screen highlighted the unexpected interaction between the regulatory subunit of the voltage–gated Ca^{2+} channels, $Ca_V\beta$, and Kir/Gem, a member of the recently identified Ras-related GTP-binding protein family RGK (**R**ad-**G**em-**K**ir). It soon appeared that all the members of this family, Gem, Rad, Rem and Rem2, were able to inhibit high-voltage activated Ca^{2+} channels, thus opening new fields of research to understand the molecular mechanisms leading to channel inhibition and to analyze their potential physiological signification. While much of these works were first concentrated on L-type $Ca_V1.2$ channels, it is clear now that presynaptic $Ca_V2.1$ and $Ca_V2.2$ channels are also sensitive to RGK inhibition. Recent data suggest that multiple routes are used by the RGK proteins to inhibit Ca^{2+} channels, including modifications of channel targeting and recycling, gating-charge mobility and/or open-channel probability. A direct RGK-$Ca_V\beta$ interaction appears to be absolutely necessary, but additional interactions with the channel protein itself have been highlighted and suggest a finely tuned specificity at the channel level. Whether these interactions also play a role in other channel $Ca_V\alpha$ or $Ca_V\beta$ functions, such as synaptic transmission or transcriptional regulation, still needs to be investigated.

Keywords RGK GTPases • Beta subunits • Trafficking • Voltage clamp • Heterologous expression

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6.1 Introduction

The inhibition of voltage-gated Ca^{2+} channels (VGCC) by trimeric GTP binding proteins Gi/Go, a crucial step for the regulation of synaptic transmission, has been the subject of important investigations for many years. By contrast, the first identification of a direct interaction between a voltage-gated Ca^{2+} channel subunit ($Ca_V\beta$) and a small monomeric GTPase Gem, belonging to the RGK Ras-related sub-family, is just a decade old (Beguin et al. 2001). This interaction has been revealed by a yeast two-hybrid analysis using the Ca^{2+} channel $Ca_V\beta_3$ subunit as bait. It promotes a strong inhibition of L-type Ca^{2+} channel activity and occurs independently of the role of the RGK on cytoskeleton remodeling that uses the RhoA-ROKβ pathways (Correll et al. 2008b). Binding to the $Ca_V\beta$ subunit and channel inhibition have been demonstrated to occur with the other members of the RGK subfamily: Rad, Rem, and Rem2, but also with the other high-voltage activated VGCC that need the auxiliary $Ca_V\beta$ subunit to be properly expressed (Rousset et al. 2005). $Ca_V1.1$, $Ca_V2.1$, $Ca_V2.2$, were thus shown to be sensitive to co-expression of the RGK GTPases while T-type Ca^{2+} channels $Ca_V3.x$ were not (Beguin et al. 2001; Chen et al. 2005; Flynn et al. 2008; Bannister et al. 2008; Leyris et al. 2009; Fan et al. 2010). However this apparent universality in the channel blocking effects of the RGK GTPases should not mask their specificity in terms of cellular expression, regulation and inhibitory mechanisms. In conjunction with the overlapping expression pattern of their potential effectors this leads to unique physiological functions. Indeed, Gem, Rad, Rem or Rem2 have been shown, in addition to their inhibitory action on VGCC, to affect synaptogenesis, cell sprouting, angiogenesis or glucose homeostasis (Pan et al. 2000; Ward and Kelly 2006; Ilany et al. 2006; Bierings et al. 2008; Correll et al. 2008b). This chapter will focus on the regulation of VGCC only.

6.2 The RGK Small GTPase Family

6.2.1 Structural Features

The RGK family comprises four members: Rad (Reynet and Kahn 1993), Gem [**G**TP binding protein induced by **M**itogens, or its mouse homolog Kir (Maguire et al. 1994)], Rem [**R**ad and G**em** related, also known as Ges, (Finlin and Andres 1997; Pan et al. 2000)] and Rem2 (Finlin et al. 2000). They have been primarily identified as transcriptionally-regulated GTPases in various tissues (Table 6.1) and in response to different stimuli (insulin, mitogene, cytokine etc.). The RGK all contain a core G domain common to the Ras-related GTPases (see Fig. 6.1), that includes five sequences (G1 to G5) involved in nucleotide and effector binding and GTP hydrolysis, but with significant non-conservative variations, as well as specific N and C-termini. The guanine binding sequences G1 ($GXG^{12}GXGKS$),

Table 6.1 Tissue-specific expression of RGK

RGK GTPAse	Tissue distribution	References
Rad	Heart, skeletal muscle, placenta, breast cancer	Reynet and Kahn (1993)
Gem	Thymus, kidney, spleen, lung, testis, monocytes fibroblasts, developing ganglia	Maguire et al. (1994) and Leone et al. (2001)
Rem	Heart, skeletal muscle, lung, kidney > brain, thymus, spleen, liver, intestine	Finlin and Andres (1997)
Rem2	**Brain**, kidney > liver≫lung heart, kidney skeletal muscle	Finlin et al. (2000)

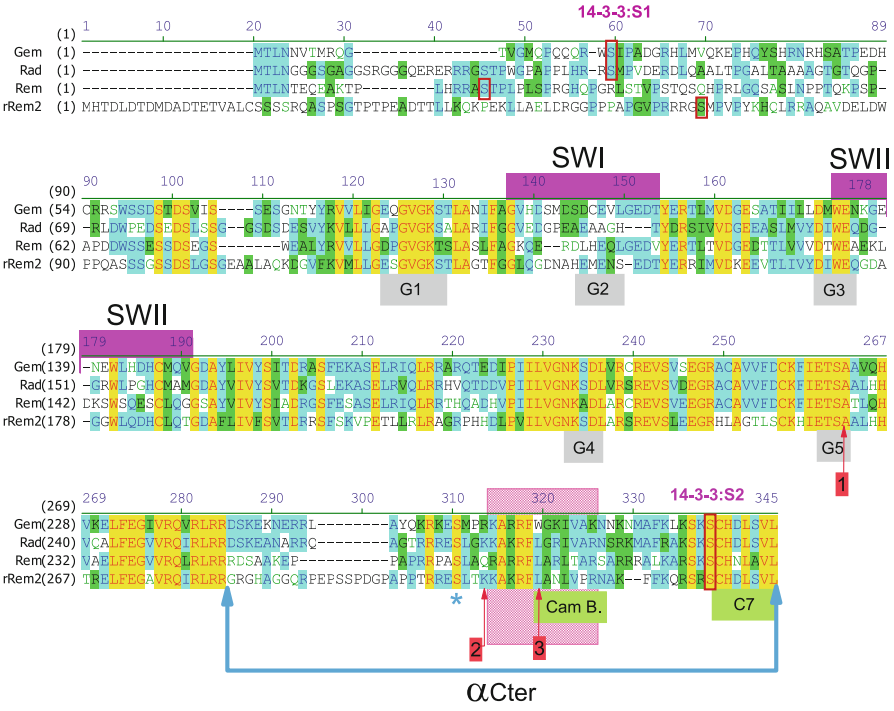


Fig. 6.1 Sequences of RGK GTPases. Aligned amino acid sequences of the four members of the RGK GTPase family Gem (Acc.nb: NM_005261), Rad (AAB17064), Rem (NM_014012) and Rem2 (XM_090793). The G domain is depicted as Rad consensus sites for phosphate binding (G1), effector binding (G2), phosphate hydrolysis and Mg binding (G3) and guanine binding (G4 and G5) domains. SWI and SWII: switch-I and switch-II regions. Cam B.: calmodulin binding site; C7: membrane targeting site. N- and C-terminal phosphorylation sites necessary for 14-3-3 mediated cytoskeleton remodeling are *boxed in red* (14-3-3S1 and S2). *Red arrow 1*: first amino acid of the C-terminal peptide (see text); *red arrow 2*: position of the deletion at aa 265 (according to Gem numbering); *arrow 3*: position of the mutation suppressing Cam binding; *pink box*: GCP1 inhibitory peptide (Fan et al. 2012). αCter: the variable C terminal α helice

G4 (NKXD) and G5 (EXSA) are quite conserved among the four RGK members.[1] The classical G3 sequence (DXXG) however, involved in GTP binding and hydrolysis, is composed of a RGK-specific consensus sequence DXWEX, and the putative G2 effector binding domain, conserved in the Ras family, which displays with the Ras proteins as well as within the RGK GTPases, important sequence variations, suggesting distinct effectors or docking sites (Finlin et al. 2000; Correll et al. 2008b). RGK also do not have the classical distal lipidation site, CAAX, present in Ras GTPases, but instead a C-terminal cysteine located within a RGK-specific sequence (C7) that follows a stretch of conserved basic residues. In addition, the presence of a Ca^{2+}-dependent calmodulin-binding (Cam–binding) domain in the C-terminus suggests specific mechanisms for membrane targeting and Ca^{2+}-dependent regulation (Fischer et al. 1996; Bilan et al. 1998). These particularities however do not obliterate the capability of RGK GTPases to bind GDP and GTP (although in the μM range) and display a high GTPase activity regulated by the N and C-termini, in a calmodulin-insensitive manner (Maguire et al. 1994; Finlin and Andres 1997; Splingard et al. 2007). In addition to their transcriptional regulation, RGK could thus also behave like molecular switches with active and inactive forms catalyzed by **G**uanine nucleotide **E**xchange **F**actors (GEF) and **G**TPases **A**ctivating **P**roteins (GAP) that have not yet been identified.

Accordingly, the analysis of the crystal structure of the G domain of Gem and Rad (Opatowsky et al. 2006; Yanuar et al. 2006; Splingard et al. 2007) underlines both similarities and differences with the other GTPases. The G-domain, involved in GTP binding, folds into canonical six stranded β sheets surrounded by five α-helices. Structural differences with the other Ras members are present in the switch-I region (that includes G2) which leaves the nucleotide pocket exposed, and in the switch-II region (containing G3), that shields the GTP γ phosphate in Ras, suggesting that the conformational changes that normally occur during the GDP/GTP cycle may be affected. In the Gem G domain structure, the proximal C–terminal sequence (before the Cam-binding site and C7) forms a highly charged α helix that makes contact with the fifth helix and the switch-II region, bringing the Cam-binding site close to core GTPase region. This conformation can also be found in Rad, but the presence of SH3 binding motifs (PxxP) in Rem and Rem2 prevents, in these two GTPases, the α-helical conformation (Splingard et al. 2007) and suggests functional differences between these GTPases.

6.2.2 Cellular Expression and Localization

Gem, Rad, Rem and Rem2 are expressed in different tissues (Table 6.1) where they are subjected to transcriptional (up or down) regulation by specific stimuli (glucose, insulin, carbachol, lipopolysaccharide, hypoxia . . .). Gem transcription for example

[1]Except for variation in G^{12}, whose mutation in Ras and Rho leads to constitutive activation, and the lack of Ras equivalent T^{35} involved in Mg^{2+}GDP/GTP binding in G1.

is up-regulated by mitogen stimulation in human T cells, by glucose in pancreatic β cells, or in neurons of tau-deficient mice (Correll et al. 2008b). Regarding inhibition of presynaptic Ca^{2+} channels, it should be noted that Rem2 was first reported to be the only member of the RGK family to be expressed at significant levels in nervous tissues (Finlin et al. 2000), but it is now admitted that Rem and Gem are also expressed in central and peripheral neurons (see localized expression on Allen Brain Atlas or BioGPS,[2] for example).

A recent study on the roles of the C-terminal tail of the RGK and various binding partners (calmodulin and 14-3-3) demonstrated that RGK localization and activity are also submitted to post-translational modification and partner regulation leading to different sub-cellular localization where regulators and/or effectors can be found. The nucleo-cytoplasmic shuttling of the RGK is under the combined control of multiple regulatory pathways via RGK phosphorylation, 14-3-3 and Cam association and lipid interaction. In the absence of Cam and 14-3-3, the presence of 3 NLS sites on Gem and interaction with importin α5 normally direct nuclear accumulation and prevent channel regulation (Mahalakshmi et al. 2007). Two serines in the N- and C-terminal tails (eq. S29 and S289 in Gem, see Fig. 6.1) of Rem, Gem, Rem2 and Rad can serve as phosphorylation-dependent docking sites for multiple members of the 14-3-3 family, binding as dimers, stabilizing the RGK structure, and leading to nuclear exclusion (Ward et al. 2004; Beguin et al. 2005a, b). This association requires the phosphorylation of the two serines and is mutually exclusive with Cam and $Ca_V\beta$ subunit binding. Phosphorylation of the C-terminus also allows interaction with the RhoA effector Rokβ, thus favouring cytoskeleton reorganisation (Ward et al. 2004). Moreover, the transient binding of Cam inhibits GTP binding and also leads to nuclear exclusion, presumably by modifying the interaction between the RGK and importin α5 (Fischer et al. 1996; Beguin et al. 2005b; Mahalakshmi et al. 2007). These mutually exclusive interactions between RGK and Cam, 14-3-3 and $Ca_V\beta$ allow the RGK to shuttle between nuclear, cytoplasmic and membrane localizations and functions (Beguin et al. 2005b). In addition, experimental evidence suggests that the phosphorylated form of Gem, cannot bind to the $Ca_V\beta$ subunit. A similar finding was found for Rad, and Rem2, whilst Rem can interact with 14-3-3 monomers by only the C-terminal binding site to promote nuclear exclusion (Beguin et al. 2006), but requires phosphorylation of both sites an 14-3-3 dimers for functional effects.

In the case of Rem2, Finlin's group has also shown that the effects on channel activity critically depend on the membrane localization of the GTPase, which relies on interactions between the C-terminal basic residues of Rem2 and membrane phosphoinositides (Correll et al. 2008a); these interactions are blocked by 14-3-3 binding.

From a functional point of view, while 14-3-3 binding does not seem to play a role in VGCC regulation, it may inhibit the cytoskeletal reorganization induced by Gem (Beguin et al. 2005b). Analysis of Cam binding-deficient RGK mutants

[2] See http://www.brain-map.org/http://biogps.org/

(mutation W269, Fig. 6.1) also suggests that, at least for Gem and Rad, the inhibitory effect of Cam on channel regulation is mainly due to its capacity to prevent nuclear sequestration of the GTPases and thus allow interaction with the $Ca_V\beta$ subunits rather than inhibiting $Ca_V\beta$ binding per se, although binding of GTP can also be inhibited by Cam (Fischer et al. 1996).

6.3 Inhibition of Ca^{2+} Channels

6.3.1 Overview of Ca^{2+} Channel Structure

Voltage-gated Ca^{2+} channels are multimeric membrane proteins composed of three major subunits forming the channel pore (the $Ca_V\alpha1$ subunit) and regulating its expression and activities[3] ($Ca_V\alpha2-\delta$ and $Ca_V\beta$ subunits, see Fig. 6.2a). Ten genes encode ten different $Ca_V\alpha1$ subunits and produce biophysically and pharmacologically distinguishable Ca^{2+} channels. They are grouped into three different families: Ca_V1, Ca_V2 and Ca_V3. Of these 3 families, only the **H**igh-**V**oltage-**A**ctivated (HVA) Ca_V1 and Ca_V2 channels (four and three members, respectively, identified as $Ca_V1.1$–$Ca_V1.4$ and $Ca_V2.1$–$Ca_V2.3$) have been shown to be tightly regulated by the auxiliary $Ca_V\beta$ and $Ca_V\alpha2-\delta$ subunits (encoded by four genes each, see Fig. 6.2b, c). Channels of the Ca_V3 family, which counts three members: $Ca_V3.1$–$Ca_V3.3$ are activated by lower membrane depolarization and termed **L**ow-**V**oltage-**A**ctivated (LVA), and function without the need for auxiliary subunits. However, the molecular architecture of all $Ca_V\alpha1$ subunits is conserved and is composed of four homologous domains (I– IV), each containing a voltage sensing region (VSR) and a pore region (PR) made of four and two transmembrane α helices, respectively, connected by intracellular loops (Fig. 6.2b) and with intracellular N and C-termini. The loop connecting domain I to II (I–II loop) beholds a conserved sequence called the α interaction domain (AID) in direct interaction with the $Ca_V\beta$ subunit, together with other secondary sequences on the intracellular C-terminal tail of the channel (Walker et al. 1998, 1999; Catterall 2011).

The four $Ca_V\beta$ subunits are cytoplasmic and constituted by the concatenation of highly conserved Src homology type 3 (SH3) and guanylate kinase (GK) domains, connected by an intramolecular hook region and non-conserved N and C terminal tails (Fig. 6.2c). A grove in the structure of the GK domain, called α binding pocket (ABP), interacts with the AID, and an additional sequence called the β interaction domain (BID) is structurally important for this interaction (De Waard et al. 1994; Rousset et al. 2005; Buraei and Yang 2010). Binding of $Ca_V\beta$ to $Ca_V\alpha1$ facilitates

[3] An additional $Ca_V\gamma$ transmembrane subunit is present in some type of Ca_V1 channels but does not appear to be essential for presynaptic Ca_V2 channels (Catterall 2011).

the trafficking of the channel to the membrane, increases its open probability and voltage-sensitivity and regulates the pharmacological properties of Ca_V1 and Ca_V2 channels (Dolphin 2003, 2012; Cens et al. 2005; Buraei and Yang 2010).

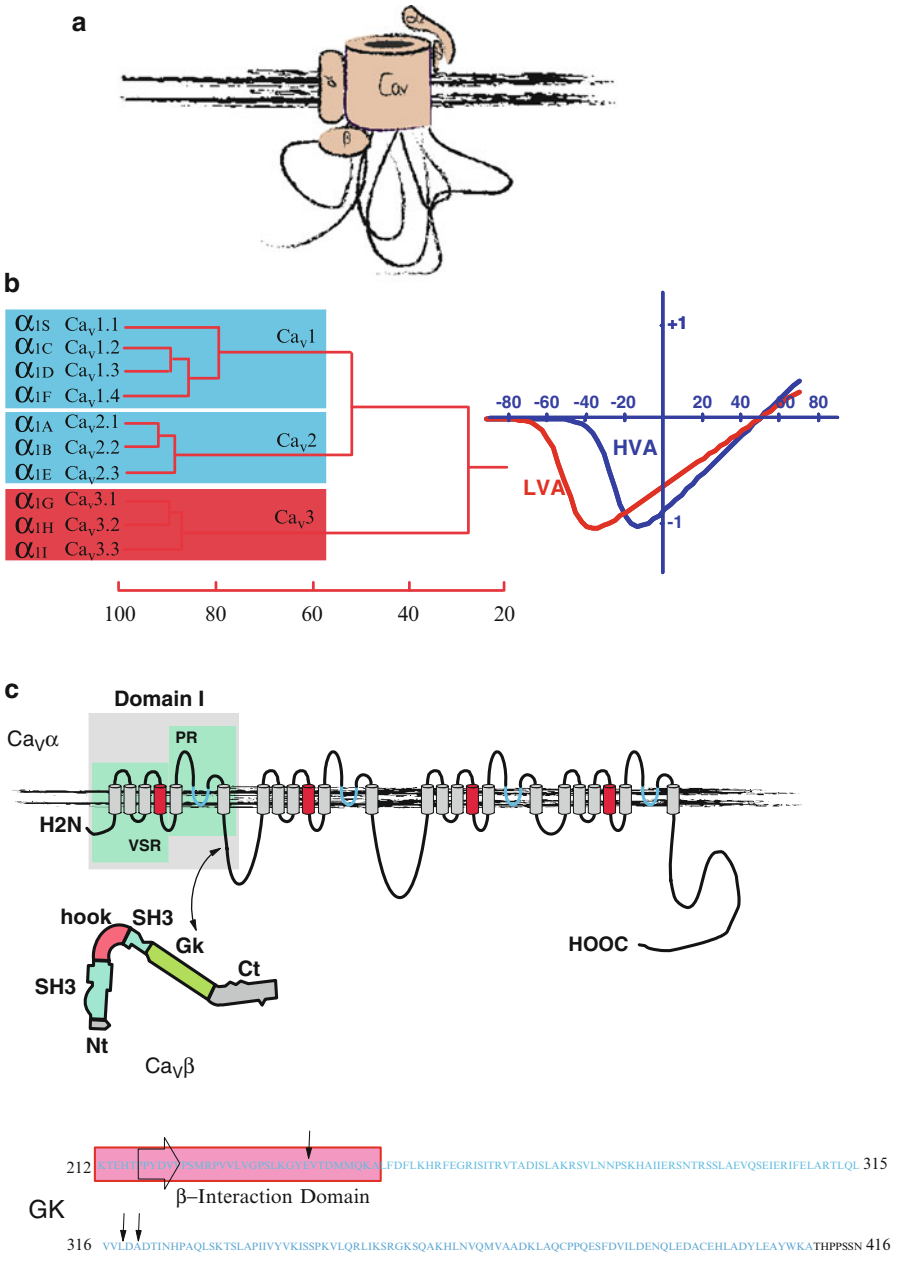
The α2−δ subunits are produced by four genes expressing pre-α2−δ proteins that are subsequently proteolyzed into two peptides, α2 and δ, that remain disulfide linked. These subunits are extracellular and glycosylated, but interact with the membrane by a short transmembrane segment of the δ subunit and/or a GPI anchor (Davies et al. 2010; Dolphin 2012).

All four $Ca_V\beta$ subunits have been shown to interact, biochemically and functionally with RGK GTPases. This interaction is GTP-dependent and does not occur with GTP-binding deficient mutants confirming that the VGCC auxiliary subunit is a *bona fide* effector of the four GTPases. However RGK binding sites have also been identified on the $Ca_V\alpha1$ channel protein on both Ca_V1 and Ca_V2 channels (see below).

6.3.2 $Ca_V1.2$ Channel Inhibition: Molecular Aspects and Regulation

Coexpression of Gem, Rad, Rem and Rem2 with the L–type $Ca_V1.2$ channels in a heterologous system completely suppresses Ca^{2+} currents. This inhibition requires the presence of the $Ca_V\beta$ subunit. The primary description of this effect suggested that the binding of GTPase with the $Ca_V\beta$ subunit disrupts the interaction between the $Ca_V\alpha1$ and $Ca_V\beta$ Ca^{2+} channel subunits thus leading to a decrease in the trafficking of channels to the plasma membrane (Beguin et al. 2001, 2005a, 2006; Sasaki et al. 2005), and in the number of gating charges [representative of the number of active channels; (Bannister et al. 2008)]. The amino acids sequence responsible for the binding of Gem onto $Ca_V\beta$ subunit (Fig. 6.2c) was localized to the beginning of the BID (see Fig. 6.2c) before the GK domain (Beguin et al. 2001; Leyris et al. 2009). The mechanisms through which this inhibition occurs is not completely understood, but may involve a GTPase-dependent nuclear sequestration of the $Ca_V\beta$ subunits (Pang et al. 2010), responsible for the reduced trafficking of $Ca_V\alpha1$ subunit to the plasma membrane, and an increase in dynamin–dependent endocytosis of the channels (Yang et al. 2010).

However, several studies have now challenged the notion that a $Ca_V\alpha1$-$Ca_V\beta$ interaction is mandatory, and even that expression of the $Ca_V\beta$ subunit is essential for the functional effects of the GTPases. The localisation of site of interaction in the $Ca_V\beta$ subunit, distant from the groove involved in the $Ca_V\beta$−$Ca_V\alpha1$ interaction, and the lack of systematic competitive interactions between these two subunits and the RGK GTPases (Beguin et al. 2007) argues indeed in favour of alternative inhibitory mechanisms. This hypothesis is reinforced by the fact that the inhibition by the GTPases can occur with $Ca_V\beta$ mutants that have lost the capacity to interact with the $Ca_V\alpha1$ subunit and/or to regulate the Ca^{2+} current amplitude (Finlin et al. 2006;


a
Cav
α
β
b
αIS CaV1.1
αIC CaV1.2
αID CaV1.3
αIF CaV1.4
CaV1
αIA CaV2.1
αIB CaV2.2
αIE CaV2.3
CaV2
αIG CaV3.1
αIH CaV3.2
αII CaV3.3
CaV3
100 80 60 40 20
+1
-80 -60 -40 -20 20 40 60 80
HVA
LVA
-1
c
Domain I
CaVα
PR
H2N
VSR
hook
SH3
Gk
Ct
SH3
Nt
CaVβ
HOOC
212 KTEHI PPYDV PSMRPVVLVGPSLKGYEVTDMMQKALFDFLKHRFEGRISITRVTADISLAKRSVLNNPSKHAIIERSNTRSSLAEVQSEIERIFELARTLQL 315
β–Interaction Domain
GK
316 VVLDADTINHPAQLSKTSLAPIIVYVKISSPKVLQRLIKSRGKSQAKHLNVQMVAADKLAQCPPQESFDVILDENQLEDACEHLADYLEAYWKATHPPSSN 416

Seu and Pitt 2006; Leyris et al. 2009). A noticeable inhibition can even take place without $Ca_V\beta$ (Crump et al. 2006), or with $Ca_V\beta$ mutants that can no longer interact with the GTPase (Yang et al. 2012).

Accordingly, interactions between Gem/Rem and the N- or C-terminus of the $Ca_V1.2$ channels have been characterized and tripartite complexes with these three full-length proteins ($Ca_V\alpha1$, $Ca_V\beta$ and RGK) may be formed. The inhibition of Ca^{2+} currents by Rem can occur without modifications in the number of active channels in myocytes, hippocampal neurons, and Min6 cells (Finlin et al. 2005; Chen et al. 2005; Xu et al. 2010), suggesting that channels can be regulated at the plasma membrane (Beguin et al. 2007; Pang et al. 2010; Yang et al. 2012). Such results are consistent with the necessary membrane localization of Rem to record the inhibitory effects on Ca^{2+} channels and its regulation by phosphorylation of the $Ca_V\alpha1$ subunit (Crump et al. 2006; Correll et al. 2007). All the above studies strongly suggest that, in addition to the perturbation in channel trafficking, several other routes can be used by the GTPases to inhibit Ca^{2+} influxes.

Indeed, GTPases can modulate both Ca^{2+} current amplitude and kinetics of activation and inactivation (Chen et al. 2005; Seu and Pitt 2006). A detailed biophysical analysis of the effects of Rem on $Ca_V1.2$ channels showed that the reduction in current amplitude involved, in addition to a decrease in channel trafficking and in a non-exclusive manner, a decrease in the channel open probability and/or immobilisation of their gating charges (Yang et al. 2010). While cytosol-targeted Rem could induce all three types of channel inhibition, membrane-targeted Rem could only decrease open probability, suggesting that different conformations of the GTPase were responsible for these effects. Moreover, if the effects on channel targeting and open-probability are strictly $Ca_V\beta$ subunit-dependent, the modulation of surface charges relies on a direct interaction between the GTPase and the channel protein itself (Yang et al. 2012), thus providing a specificity toward different channel types, with $Ca_V\beta$ subunit-dependent and independent inhibition. Although this has not been systematically investigated for all the $Ca_V\alpha1$-RGK pairs, Rem2 and Rad have also been shown to interact with a proximal region of the C-terminus of $Ca_V1.2$ channels, that contains a calmodulin IQ binding-site and

Fig. 6.2 Voltage-gated Ca^{2+} channels: classification and subunit structures. (**a**) Schematic representation of the multimeric Ca channel structure with the $Ca_V\alpha1$, $Ca_V\alpha2-\delta$ and $Ca_V\beta$ subunits. (**b**) Classification and nomenclature of the $Ca_V\alpha1$ subunits grouping the subunits into three main families according to sequence homology. *Right*, exemplar current-voltage curves for HVA (Ca_V1 and Ca_V2, in *blue*) and LVA (Ca_V3, in *red*) Ca^{2+} channels. (**c**) Schematic representation of the membrane topography, domain structure and inter-subunit interaction of $Ca_V\alpha1$ and $Ca_V\beta$ subunits. PR: pore region, VSR, voltage-sensing region. SH3: src-homology type 3 region, GK: guanylate kinase region, Nt, Ct: variable N- and C-termini, respectively. (**d**) Sequence of the guanylate kinase (GK) domain of the $Ca_V\beta_2$ subunit (*in blue*). The *open arrow* depicts the last SH3 α helice that is separated from the four others by the hook region, and the *pink box* shows the BID sequence. *Black arrows* shows the mutations that inhibit the interaction with the RGK (Beguin et al. 2007; Yang et al. 2012)

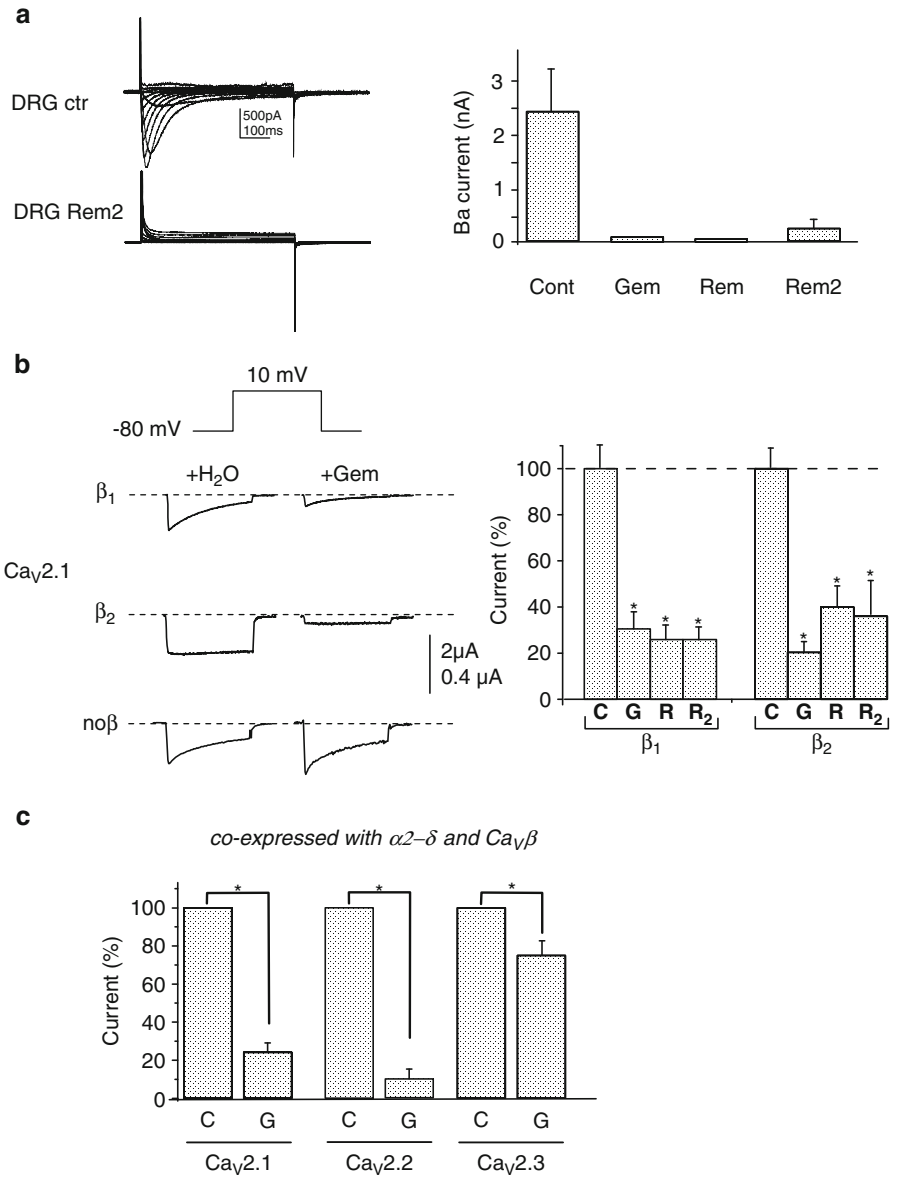


Fig. 6.3 Inhibition of $Ca_V2.x$ Ca^{2+} channels by RGK GTPases. (**a**) Effect of electroporation of Gem, Rem and Rem2 on DRG neurons in primary culture. *Left*: typical Ba^{2+} current traces recorded during depolarization to +10 mV from a holding potential of −80 V on DRG neurons electroporated with control (ctr) or Rem2 plasmids. *Right*: averaged effects of the electroporation of Gem, Rem, Rem2 or control plasmid (Gem, Rem, Rem2 or cont, respectively) on global current amplitude. (**b**) Inhibition of $Ca_V2.1$ Ca^{2+} channels by Gem is $Ca_V\beta$ subunit-dependent. *Left*: current traces recorded on different *Xenopus* oocytes expressing the $Ca_V2.1$ Ca^{2+} channel subunit and the $Ca_V\alpha2-\delta$ with $Ca_V\beta_1$, $Ca_V\beta_2$ or no $Ca_V\beta$ subunit.

regulates channel inactivation in a calmodulin-sensitive manner (Pang et al. 2010). Moreover, Rem also interacts with the N-terminus of $Ca_V1.2$, and this interaction drives $Ca_V\beta$ subunit-independent decrease in surface charges mobility, but not surface channel density (Yang et al. 2012). The use of this N-terminal ($Ca_V\beta$ independent) site is however only effective for Rem and Rad. All these results provide an unexpected variation in the palette of possible $Ca_V\beta$ subunit-dependent and independent interactions and regulations between different RGK and $Ca_V\alpha1$ subunits that should ensure a finely tuned regulation in physiological situations. The situation is even more complex if one considers the existence of splice variants of the $Ca_V\alpha1$ subunits in the region that have been shown to interact with the RGK (N and C terminus). However, the impact of this splicing on RGK functions is unknown. It should be noted that, although this has not been systematically tested, the effect of $Ca_V1.2$ by RGK did not appear to be sensitive to the $Ca_V\alpha2{-}\delta$ subunit since inhibition has been recorded with (Seu and Pitt 2006) or without (Beguin et al. 2001) expression of the regulatory subunit.

6.3.3 $Ca_V2.x$ Channels Inhibition: Similarities and Specificities

The literature concerning RGK inhibition of presynaptic Ca_V2 VGCC is far less rich than that for $Ca_V1.2$. The first evidence of their inhibition came from studies on endogenous HVA channels in sympathetic and dorsal root ganglion (DRG) neurons. In DRG neurons, over-expression of Gem, Rem or Rem2 almost completely blocks all HVA channels (see Fig. 6.3b), which are composed principally of $Ca_V1.2$, $Ca_V2.1$, $Ca_V2.2$ and to a lesser extent $Ca_V2.3$ subunits (Murakami et al. 2001). Further analysis of the inhibition of Ca_V2 channels highlighted two specificities (1) channels could be blocked by Rem2 without modifications in the number of channels at the plasma membrane (Chen et al. 2005) and (2) this inhibition absolutely required the presence of a $Ca_V\beta$ subunit (at least for $Ca_V2.1$ and $Ca_V2.2$) and the Rem2 polybasic C terminus (Flynn et al. 2008), indicating a plasma membrane mechanism leading to blockade of pre-existing channels. Indeed, the $Ca_V2.2$ channel lacks the N-terminal interaction site for GTPases that allows the $Ca_V\beta$ subunit-independent blockade of gating charges in $Ca_V1.2$ (Yang et al. 2012).

In expression systems, $Ca_V2.1$, expressed with $Ca_V\beta_1$ or $Ca_V\beta_2$, has also been reported to be sensitive to Gem, Rem and Rem2 (Beguin et al. 2001; Leyris et al. 2009; Fan et al. 2010). As for $Ca_V2.2$, this inhibition was strictly $Ca_V\beta$-dependent

Fig. 6.3 (continued) Coexpression of Gem in these different oocytes can block Ca influx only when a $Ca_V\beta$ subunit is expressed. *Right*: Mean effect on current amplitude of coexpressing Gem (G), Rem (R1) or Rem2 (R2) with the $Ca_V2.1{+}Ca_V\alpha2{-}\delta$ subunit and $Ca_V\beta_1$ or $Ca_V\beta_2$. C: control current without expression of the GTPase. (**c**) Mean normalized effect on current amplitude of the expression of Gem (G) on oocytes expressing either, $Ca_V2.1$, $Ca_V2.2$ or $Ca_V2.3$ with $\alpha2{-}\delta$ and $Ca_V\beta_2$. C: control without Gem

(Leyris et al. 2009), but a direct RGK-$Ca_V\beta$-interaction and $Ca_V\beta$-induced current potentiation seemed dispensable for current inhibition (Leyris et al. 2009; Fan et al. 2010). A direct interaction between the RGK and the $Ca_V2.1$ subunit, delimited to the transmembrane S1, S2 and S3 segments (Fig. 6.2c) of the second domain of the subunit, was reported to be necessary, albeit not sufficient, for channel modulation.

It is worth noting that, in expression systems, a complete blockade of $Ca_V2.1$ or $Ca_V2.2$ channels was never obtained (see Fig. 6.3c; Beguin et al. 2001; Chen et al. 2005; Leyris et al. 2009), with a residual current showing all the biophysical hallmarks of $Ca_V\beta$-containing channels. These resistant channels have not been studied in detail, but suggest that additional regulatory factors and/or partners may dictate channel sensitivity to RGK.

The emerging concept of these studies proposes therefore that Ca^{2+} channels need to be primed at the plasma membrane by the $Ca_V\beta$ subunit, before being able to functionally respond to $Ca_V\alpha1$-RGK interactions. Interestingly, the group of Dr. Yang delineated precisely two additional sites in the RGK Gem, a 12 amino-acid peptide in the C-terminus (GCP1 in Fig. 6.1) and a 3 amino-acid motif in the G domain, that were found to be critical for this $Ca_V\beta$-dependent inhibition but not involved in a direct interaction with $Ca_V\alpha1$ or $Ca_V\beta$, suggesting the existence of inhibitory sites in addition to the interacting sequences (Fan et al. 2012). In these experiments intracellular perfusion of GCP1 was sufficient to produce a 80 % channel block, consistent with previous experiments where a C-terminal fragment (starting at arrow 1 in Fig. 6.1) was also sufficient for efficient inhibition (Leyris et al. 2009).

The effects of RGK on the $Ca_V2.3$ Ca^{2+} channels have not been directly reported yet. However, in DRG neurons known to express a R-type current (resulting from $Ca_V2.3$ expression), the complete block of the HVA Ca^{2+} currents following over-expression of the GTPase Gem or Rem strongly suggested that $Ca_V2.3$-Rtype channels are also sensitive (Fig. 6.3a). Our comparative work on the inhibitory effects of Gem on $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$, co-expressed with $Ca_V\alpha2{-}\delta$ and $Ca_V\beta2$ in Xenopus oocytes, however evidenced subtle differences between the various $Ca_V\alpha1$ subunits, $Ca_V2.3$ being less sensitive (work in preparation, see Fig. 6.3c). However, the role of the $Ca_V\beta$ subunit in this inhibition, and the potential direct interaction between $Ca_V2.3$ and RGK remains to be determined.

Finally, the identification of multiple specific interactions and inhibitory sites on RGK, $Ca_V\beta$ and $Ca_V\alpha1$ subunits is thus conceptually in phase with the multiple mechanisms used by RGK to inhibit the different types of Ca^{2+} channels. Taken together, the studies in native and heterologous systems underline the potential crucial importance of RGK to tune the Ca^{2+} response to different physiological situations.

6.4 Physiological Implications

RGK GTPases are involved in a number of signalling pathways in neuronal and non-neuronal tissues. Modulation of the cytoskeletal dynamics via inhibition of the RhoA-ROKβ or interaction with kinesin-like proteins such as KIF9

(Piddini et al. 2001) pathways leads to modification of cell shape, neurite extension, formation of filopodia, disassembly of stress fibers etc. [see (Ward and Kelly 2006) for review] in various cell types. In cardiac myocytes, down regulation of Rad or Rem GTPases induces cardiac arythmias linked to increased Ca^{2+} currents, suggesting a role of the GTPase in vivo (Yada et al. 2007; Magyar et al. 2012). By their action on $Ca_V1.2$ Ca^{2+} channels, Rem, Rem2, Rad or Gem also blocks excitation-contraction coupling in skeletal muscle or glucose-induced insulin secretion in pancreatic β cells (Finlin et al. 2005; Bannister et al. 2008; Gunton et al. 2012).

In neuronal tissue, in addition to the effect on neurite extension, RGK (Rem2) act in concert with other GTPases (e.g. RhoA, CDC42) to regulate early synapse development, affecting neuron arborisation and branching by interconnected signalling pathways (Paradis et al. 2007; Ghiretti and Paradis 2011). These effects have been linked to neuronal Ca^{2+} homeostasis. A subsequent study, testing the potential effect of RGK-induced Ca^{2+} channel inhibition on hippocampal synapse development and activity, was not able to define the role of Rem2-induced Ca^{2+} channel regulation in these processes (Wang et al. 2011). However, these physiological studies are at an early stage, and many situations where both GTPase and Ca^{2+} concentration are required (e.g. axon pathfinding, regeneration) will need to be investigated to get a clearer picture of the physiological relevance of these regulations. Indeed the generation of Gem and Rem knock-out mice will be important tools for these studies, even if their preliminary analysis suggests that compensatory mechanisms may exist (Gunton et al. 2012; Magyar et al. 2012).

6.5 Possible Crosstalks with Other $Ca_V\alpha/Ca_V\beta$-Dependent Regulations or Functions

It is worth-noting that the last years have revealed new roles for $Ca_V\alpha1$ and $Ca_V\beta$ subunits in addition to their Ca^{2+}-passing functions. $Ca_V1.2$ or $Ca_V2.1$ subunits, either via Ca^{2+} flowing through their pore or via their cleavable C-termini, can send messengers to the nucleus and regulate transcription (Dolmetsch 2003; Kordasiewicz et al. 2006; Gomez-Ospina et al. 2006; Wheeler et al. 2012). This C-terminus is also responsible for $Ca_V1.2$ channel regulation by PKA-dependent phosphorylation (Fu et al. 2011; Marshall et al. 2011). $Ca_V\beta$ subunits are known to be addressed to the nucleus and regulate transcription (see Hibino et al. 2003; Zhang et al. 2010; Tadmouri et al. 2012). Thus, the finding that RGK can traffic, in a Cam and 14-3-3-dependent manner, $Ca_V\beta$ subunits to the nucleus or interact with C-termini of $Ca_V\alpha1$ subunits suggests that they may regulate their nuclear functions. A number of new partners for the $Ca_V\alpha1$, $Ca_V\alpha2{-}\delta$ or $Ca_V\beta$ subunits, [Rim1, Ahnak, Ryanodine receptors, Best1, K^+ channels etc. (Buraei and Yang 2010; Muller et al. 2010)], affecting channel kinetics or Ca^{2+} signalling functions, have expanded the number of signaling pathways connected to Ca^{2+} channels and constitute therefore additional potential targets for RGKs (see Fig. 6.4). RGK may also compete with other small GTPase-dependent signalling; for example, RhoA

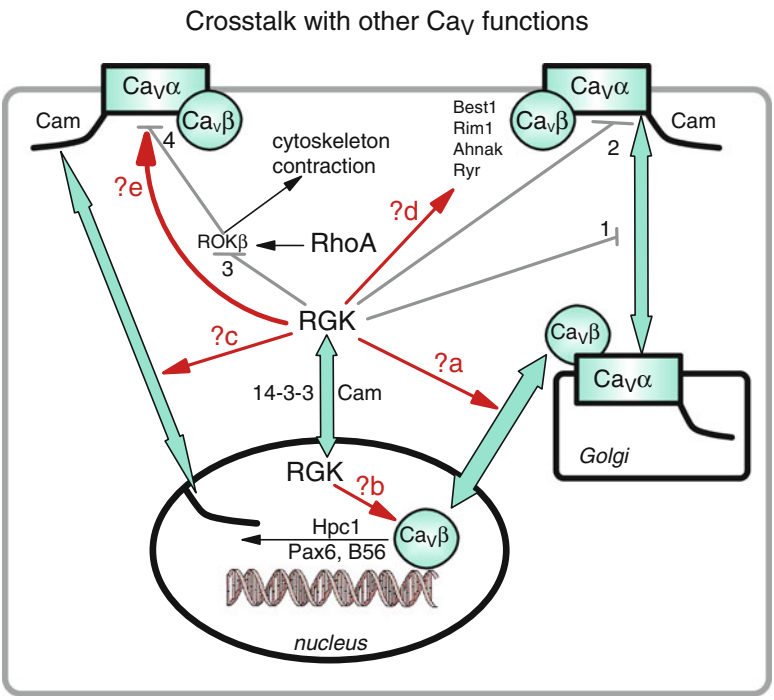


Fig. 6.4 Cross-talk with other Ca_V functions. (**a**) non-exhaustive schematic representation of the demonstrated and other potential effects of RGK GTPases on Ca_V functions. Only $Ca_V\alpha1$ and $Ca_V\beta$ subunits are represented. RGK have been shown to inhibit translocation of the HVA Ca^{2+} channel to the plasma membrane (*grey line 1*), and to decrease activity (open probability, voltage sensor mobility, gating, see text) of channel at the plasma membrane (*grey line 2*). RGK have also been shown to block the effect of the RhoA-activated ROKa kinase on cytoskeleton reorganization, and smooth muscle contraction (*grey line 3*). Additionally, RhoA/ROKa also regulates LVA and HVA channels (*grey line 4*). However the effects of the RGK on other $Ca_V\alpha1$ or $Ca_V\beta$ functions have not been analyzed (*identified as red arrows with a question mark*). (1) The effects of RGK on the nuclear translocation of $Ca_V\beta$ (*?a*) and the $Ca_V\beta$-dependent regulation of transcription (*?b*) awaits experimental testing. (2) The C-terminus of $Ca_V1.2$ can be cleaved and regulate channel activity or be translocated to the nucleus and regulate transcription. The consequences of the binding of RGK on $Ca_V\alpha1$ and/or $Ca_V\beta$ on these mechanisms have not been studied (*?c*). (3) Multiple partners can bind $Ca_V\beta$ and regulated Ca^{2+} channel activity and synaptic transmission in neurons or contraction in muscle cells (Best 1, Rim1, Ahnak, Ryanodine receptor for example). How the binding of RGK on $Ca_V\alpha1$ and/or $Ca_V\beta$ affects these regulations is not known (*?d*). (4) RhoA-activated ROKβ kinase can phosphorylate $Ca_V\alpha1$ subunit and modify channel activity, but the potential crosstalk of this regulation with the RGK pathway on channel activity has not been studied yet (*?e*)

has been shown to regulate both LVA and HVA Ca^{2+} channels via Gem-sensitive ROK-dependent phosphorylation (Ward et al. 2002; Iftinca et al. 2007). How the $Ca_V\alpha1-$ or $Ca_V\beta-$RGK interactions modify these pathways will now need to be tested in defined conditions, but will surely reveal new aspects of the RGK-VGCC interactions.

6.6 Conclusions

In-depth analysis of the literature on the regulation of Ca^{2+} channels by RGK, often leads to a confused picture, where the effects GTP, Cam, 14-3-3 binding on the GTPase/$Ca_V\beta$ and/or $Ca_V\alpha1$ interactions and the functional effects on Ca^{2+} entry can be contradictory. However, these studies have been conducted in different systems, using different sets of subunits and RGK, sometimes from different species. Variations in the relative expression of these factors in different cell types may also explain these specific cellular localizations, interactions and regulations. Indeed, starting from a simple RGK/$Ca_V\beta$ subunit interaction able to lock Ca^{2+} channel trafficking, a decade of intense experimental work has revealed the huge potential offered by the RGK to regulate channel function in various situations with triple specificity ($Ca_V\alpha1$, $Ca_V\beta$, RGK) and a multiplicity of the inhibitory mechanisms. This complexity appears to be well suited for fine regulation of Ca^{2+} homeostasis. However, while the complete understanding of these mechanisms in vivo still requires extensive experimental work, their functional effects are already used to conceive a new generation of inducible Ca^{2+} channel blockers with exquisite specificity (Murata et al. 2004; Yang et al. 2007; Xu and Colecraft 2009), and with the potential to surpass classical pharmacological approaches, at least for fundamental research.

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Chapter 7
Protein Interaction Partners of $Ca_v2.3$ R-Type Voltage-Gated Calcium Channels

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Abstract The $Ca_v2.3$ voltage-gated calcium channel represents the most enigmatic of all voltage-gated calcium channels due to its pharmacological inertness and to its mixed characteristics of HVA and LVA calcium channels. Protein interaction partners of the cytosolic II-III linker of $Ca_v2.3$ contribute to calcium homeostasis by regulating the channels surface expression and activation. Specific regulation of $Ca_v2.3$ by proteins interacting with the carboxy terminal region plays an important role in exocytosis and presynaptic plasticity, linking channel function to long-term potentiation. Modulation of $Ca_v2.3$ by its interaction partners thus contributes

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to several physiologic processes such as signal transduction in the retina, insulin secretion and generation of rhythmic activity in the heart and in the brain.

Keywords $Ca_v2.3$ • R-type current • Amyloid precursor protein • Calmodulin • Rab5A • Heat shock proteins vacuolar ATPase

Abbreviations

APP	Amyloid precursor protein
APLP1	Amyloid precursor-like protein 1
CaM	Calmodulin
CDF	Calcium-dependent facilitation
CDI	Calcium-dependent inactivation
DHP	Dihydropyridines
EGFR	Epidermal growth factor receptor
HVA	High-voltage activated
LVA	Low-voltage activated
PKC	Protein kinase C
V-ATPase	Vacuolar ATPase
VGCC	Voltage-gated calcium channel

7.1 Introduction

7.1.1 Voltage-Gated Calcium Channels

Voltage-gated calcium channels (VGCCs) are expressed on the plasma membrane of excitable cells, where they regulate calcium ion permeability. Calcium ions are the most versatile second messengers and also serve as charge carriers. VGCCs respond to changes in membrane potential and convert cellular electrical excitability into intracellular signaling. Calcium channels are multi-subunit integral membrane proteins, with a large (>250 kD) pore-forming and voltage-sensing α1 subunit and smaller auxiliary transmembrane α2δ and cytoplasmic β subunits (Catterall 2011). The auxiliary β subunits regulate proteasomal degradation of the α1 subunit (Altier et al. 2011; Rougier et al. 2011 Waithe et al. 2011) making them crucial for cellular trafficking and stable surface expression of the α1 subunit. The function of plasma membrane calcium channels can be critically modulated by various signaling pathways, and frequently involves transient or persistent interaction of certain cellular proteins with the α1 subunit. These interactions also tightly regulate the amount of calcium channels expressed on the cell surface. These processes are important because small changes in the number of surface channels can greatly affect cell signaling.

Based on their biophysical and pharmacological properties, VGCCs can be classified into three groups. (i) L-type high-voltage-activated (HVA) calcium channels comprising the $Ca_V1.1$, 1.2, 1.3, and 1.4 channels, which can be inhibited by dihydropyridines (DHPs), phenylalkylamines and benzodiazepines (Striessnig 1999; Catterall and Few 2008; Dolphin 2009), (ii) non-L-type HVA channels $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type), and $Ca_V2.3$ (R-type) that are sensitive to ω-agatoxin IVA and ω-conotoxin GVIA and SNX 482, respectively (Reid et al. 2003; Kamp et al. 2005; Catterall and Few 2008), and (iii) the low-voltage-activated (LVA) T-type calcium channel family ($Ca_V3.1$, 3.2, and 3.3) (Perez-Reyes 2003). Among the calcium channels, the R-type/$Ca_V2.3$ calcium channel has been less explored due to its pharmacological inertness (it's only known peptide inhibitor SNX-482 also antagonizes L- and N-type calcium channels at concentrations higher than 300 nM). Under the perforated patch configuration it has been shown in chromaffin cells that 20 % of I_{Ca} can be accounted for by toxin-resistant, R-type calcium currents (Albillos et al. 2000; Hernandez et al. 2011).

7.2 Discovery of R-Type/E-Type Voltage-Gated Calcium Channels

The first evidence for increased structural diversity of high-voltage gated calcium channels came from the cloning of new calcium channel types from the rabbit brain (Niidome et al. 1992) and from the forebrain of the marine ray *Discopyge ommata* (Horne et al. 1993). The complete amino acid sequence from rabbit, designated BII, showed structural similarity to the so-called BI sequence, encoding the non-L-type voltage-gated calcium channel i.e. the P-/Q-type calcium channel (Mori et al. 1991). Transcripts of BII were predominantly identified in the brain and most abundant in the cerebral cortex, the hippocampus and the corpus striatum (Niidome et al. 1992).

The consecutive approach to identify calcium current components homologous to the ray doe-1 channel in the CNS of mammalia was successful for rat cerebellar granule cells (Zhang et al. 1993). Doe-1 formed high voltage-activated calcium currents when expressed in *Xenopus* oocytes, and inactivated more rapidly than any of the previously identified calcium channels. The high voltage-activated Ca^{2+} current component, which persisted after blocking L-, N- and P-/Q-type calcium channels, was defined as the "resistant"/R-type voltage-gated Ca^{2+} current (Ellinor et al. 1993; Zhang et al. 1993). The mammalian counterpart of doe-1 was cloned from rat (Soong et al. 1993) and finally also from human (Williams et al. 1994) and was occasionally referred to as the "E-type" voltage-gated calcium channel (Schneider et al. 1994). After functional expression of the rat $Ca_v2.3$ clone, it was speculated that this channel may represent the low voltage-activated T-type calcium channel, which at that time had yet to be structurally identified (Soong et al. 1993). Instead, cloning and expression of human and rabbit $Ca_v2.3$ splice variants

revealed a high-voltage-activated calcium channel (R-type), at least in heterologous expression systems (Schneider et al. 1994; Wakamori et al. 1994; Williams et al. 1994).

The R-type calcium channel received its name from "resistant" and indeed to date no highly selective antagonists exist. SNX-482, a toxin found in the venom of the tarantula *Hysterocrates gigas* does show selectivity for R-type channels but also inhibits L-type and N-type channels at concentrations beyond 200 nM (Bourinet et al. 2001). Although the structure of $Ca_v2.3$ deduced from sequencing of cDNA has been known for several years (Perez-Reyes and Schneider 1994; Pereverzev et al. 2002a), its physio- and pathophysiological role remain only partially recognized (Kamp et al. 2005; Weiergräber et al. 2006). Evidence suggests that $Ca_v2.3$ developed very early in evolution (Zhang et al. 1993; Perez-Reyes 2003; Spafford and Zamponi 2003), which may underline its great significance in vivo.

In heterologous expression systems, $Ca_v2.3$ inward currents are activated at test potentials of about –20 mV (De Waard et al. 1996). The single channel conductance is about 14 pS (Perez-Reyes and Schneider 1995), and the channel kinetics measured by patch-clamp recordings reveal a fast activating and inactivating channel type with transient inward current characteristics (Pereverzev et al. 2002a; Leroy et al. 2003), similar but not as fast as observed for T-type voltage-gated calcium channels (Nakashima et al. 1998).

7.3 Expression of $Ca_v2.3$ Voltage-Gated Calcium Channels in Various Regions of the Vertebrate Organism

The $Ca_v2.3$ VGCC is widely expressed throughout the vertebrate organism, not only in the central nervous system (for details, see Table 2 in Kamp et al. 2012b) Its initial detection in the endocrine system of mice and rats (Pereverzev et al. 2002b, 2005; Jing et al. 2005; Trombetta et al. 2012) was recently confirmed for the human organism as well (Muller et al. 2007; Trombetta et al. 2012). Endothelial and myocardial expression of R-type calcium channels (Lu et al. 2004; Weiergräber et al. 2005; Galetin et al. 2010) has been well established on a transcriptional and functional level, however, detecting myocardial $Ca_v2.3$ protein has proven to be problematic (Tevoufouet and Schneider, unpublished results). Interestingly, $Ca_v2.3$ is also expressed in the reproductive system (Sakata et al. 2002) and the gastrointestinal tract (Grabsch et al. 1999; Naidoo et al. 2010), where in the latter case its functional importance during autonomous excitation generation must be analyzed in greater detail. More recently, the involvement of R-type calcium channels in delayed cerebral ischemia has been shown in animal models of subarachnoid haemorrhage, in which blood metabolites induce expression of R-type calcium channels in cerebral arteries (Ishiguro et al. 2008; Wang et al. 2010). Furthermore, the subcellular distribution of $Ca_v2.3$ has been investigated to some detail revealing both, somatodendritic as well as presynaptic expression (Yokoyama et al. 1995) with additional functional specificities (Brenowitz and Regehr 2003).

7.3.1 *Expression of $Ca_v2.3$ Splice Variants*

Originally, $Ca_v2.3d$ was cloned as a fetal splice variant from human brain (Schneider et al. 1994). Splice variants of $Ca_v2.3$ from different species as well as auxiliary subunits are tissue-specifically expressed. Besides the expression in neuronal (Han et al. 2002; Sochivko et al. 2002, 2003; Dietrich et al. 2003; Osanai et al. 2006) and endocrine tissues (Vajna et al. 1998; 2001; Grabsch et al. 1999; Wang et al. 1999; Albillos et al. 2000; Matsuda et al. 2001; Pereverzev et al. 2002b; Mergler et al. 2003; Watanabe et al. 2004; Jing et al. 2005; Ortiz-Miranda et al. 2005; Pereverzev et al. 2005; Holmkvist et al. 2007; Muller et al. 2007; Zhang et al. 2007), $Ca_v2.3$ transcripts have also been detected in heart (Weiergräber et al. 2000, 2005; Lu et al. 2004), kidney (Vajna et al. 1998; Schramm et al. 1999; Weiergräber et al. 2000; Natrajan et al. 2006), sperm (Lievano et al. 1996; Wennemuth et al. 2000; Sakata et al. 2002; Carlson et al. 2003), spleen (Williams et al. 1994), and retina (Kamphuis and Hendriksen 1998; Lüke et al. 2005) (for details, see Table 2 in Kamp et al. 2012b).

Structurally, a broad set of $Ca_v2.3$ splice variants can be predicted from different cloning approaches (Fig. 7.1) resulting from alternate use of exon 19 encoded arginine-rich segment in the II-III loop, as well as from the alternate use of exon 45 in the carboxyterminal region (Pereverzev et al. 2002a).

7.4 Structure and Function of the $Ca_v2.3$ Voltage-Gated Calcium Channel

The complete quaternary structure of native VGCCs containing $Ca_v2.3$ is unknown, but may resemble purified calcium channel complexes (Perez-Reyes and Schneider 1994) and thus may contain additional subunits including the well known auxiliary $Ca_v\beta$-subunits, which modulate $Ca_v2.3$-mediated inward currents in heterologous expression systems (Parent et al. 1997; Nakashima et al. 1998). To date, VGCCs containing $Ca_v2.3$ have not been purified as has been accomplished for L-type calcium channels from rabbit skeletal muscle (Flockerzi et al. 1986; Sieber et al. 1987; Takahashi et al. 1987; Striessnig et al. 1987), and bovine heart (Schneider and Hofmann 1988) and for the neuronal N-type calcium channels (Witcher et al. 1993a, b).

Sequence comparison of the deduced primary sequence revealed a well known intra-molecular homology pattern, which is found in all VGCCs as well as in voltage-gated Na^+ channels. This pattern contains four internal repeats, which have been termed domains I, II, III, and IV. Secondary structure analysis predicts 6 transmembrane segments including a random coiled short part between transmembrane segment 5 and 6, the pore forming segment (P-loop) (Guy and Conti 1990). Many of these structure predictions resemble the confirmed structural elements in the bacterial and rat voltage-gated K^+-channel (Doyle et al. 1998; Long et al. 2005).

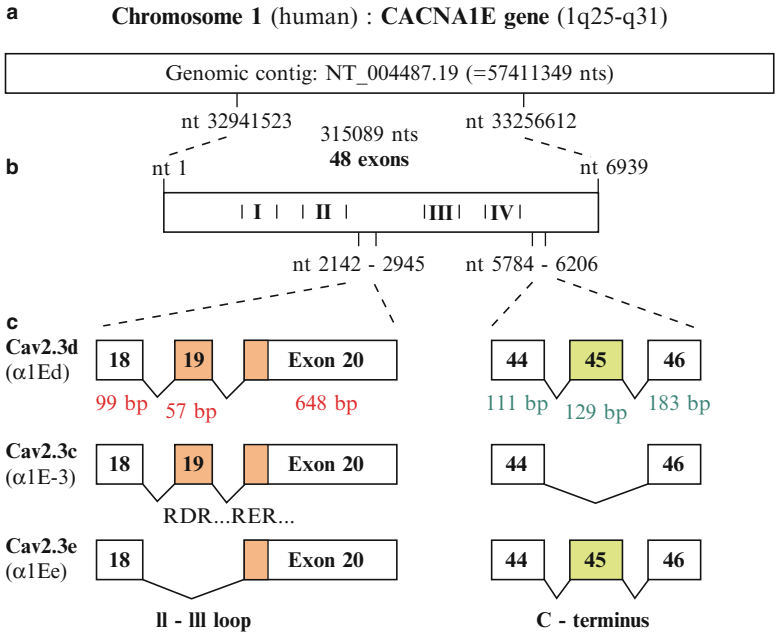

Fig. 7.1 Splice variants of $Ca_v2.3$ (alternative skipping of exon 19 and exon 45). Partial intron-exon structure of the human $Ca_v2.3$ subunit demonstrates the major splice variants reported in the literature (for details see: Pereverzev et al. 2002a). (**a**) The human gene of $Ca_v2.3$ is located on chromosome 1. Aligning the cloned human cDNA (GenBank L27745) to the Human Genome data bank led to the detection of the contig NT_004487.19, with a length of 54,411,349 nucleotides. The human cDNA aligned to the region between nt 32,941,523 and nt 33,256,612 within this contig and comprising 48 exons. (**b**) Two regions of the $Ca_v2.3$ subunit were investigated for structural variations, the II–III linker, containing exon 18 to exon 20 at the position 2142–2945, and the carboxy terminus, showing exon 44 to exon 46 at the position 5784–6206 of the human $Ca_v2.3$ cDNA. (**c**) Three major splice variants of the mammalian $Ca_v2.3$ subunit have been determined in vivo. The splice variant which was cloned from human fetal brain contains both exon 19 and exon 45 ($Ca_v2.3d$). The neuronal $Ca_v2.3c$ and the endocrine $Ca_v2.3e$ splice variant lack exon 19 and exon 45, respectively. The 57 nt of exon 19 encode an arginine-rich region similar to the first 19 aa of exon 20, which is shown by the first three amino acids of each segment (RDR . . . and RER . . .)

Additional elements may contribute to the kinetic properties of $Ca_v2.3$-mediated inward currents as reported for structurally similar ion channels. The segments S6 participate in gating the ion channels (Hofmann et al. 1999; Zhen et al. 2005; Xie et al. 2005), and the P-loops form essential components of the selectivity filters, thus also influencing the speed of the ion flux through the pore (Kim et al. 1993; Tang et al. 1993; Yang et al. 1993; Ellinor et al. 1995; Parent and Gopalakrishnan 1995; Dirksen et al. 1997; Cibulsky and Sather 2000; Cibulsky and Sather 2003). The segment S4 acts mainly as the voltage sensor (Jiang et al. 2003; Lacinova 2005), and its detailed orientation to the pore region has been elucidated in crystals from bacterial K^+ and Na^+ channels to a great extent (Lee et al. 2009; Payandeh

et al. 2011). Furthermore, mutational analysis revealed that separate regions of $Ca_v2.3$, like the conserved hydrophobic locus VAVIM in the S6 transmembrane segment of domain IV, are involved in voltage-dependent gating (Raybaud et al. 2007). Hydrophobic residues in the VAVIM locus (and other residues) promote the channel's closed state rendering them critical for the stability of the channel's closed and open states. Additionally, mutational analysis of a leucine residue in S4S5 provides the first evidence that the IIS4S5 and the IIS6 regions are energetically coupled during the activation of a VGCC (Wall-Lacelle et al. 2011).

7.5 Interaction Sites of $Ca_v2.3$ Voltage-Gated Calcium Channels

Interactions of $Ca_v2.3$ with its few known interaction partners have yet to be visualized by crystallization, but have been modeled (e.g. interaction with $Ca_v\beta$-subunits (Berrou et al. 2005)) and investigated in heterologous expression systems (Krieger et al. 2006). The interaction site of $Ca_v\beta$ with $Ca_v1.1$ and $Ca_v1.2$ is located in a conserved region between domain I and II (De Waard et al. 1994; Pragnell et al. 1994), which also contains the interaction site of $Ca_v2.3$ with $Ca_v\beta$-subunits (Berrou et al. 2001, 2005). The affinity of G-protein $\beta\gamma$ complexes towards the $Ca_v2.3$ I-II loop is similar as towards the I-II loops of the related $Ca_v2.1$ and $Ca_v2.2$ $\alpha1$-subunits, which are all three six to eight-fold higher as towards L-type $\alpha1$ subunits (De Waard et al. 1997).

Segments of the cytosolic loops of $Ca_v1.2$ L-type calcium channels have been co-crystallized with functional auxiliary subunits of VGCCs (Van Petegem et al. 2004) or functionally interacting calmodulin (Petegem et al. 2005; Dick et al. 2008; Kim et al. 2008; Tadross et al. 2008). For $Ca_v2.3$ this interaction was compared and predicted by modelling. Molecular replacement analyses were carried out using a three-dimensional homology model for the AID with the auxiliary $Ca_v\beta$-subunits (Berrou et al. 2005). Together with other data (Van Petegem et al. 2004), these results revealed detailed information about how the AID may functionally interact with $Ca_v\beta$-subunits in high voltage-activated calcium channels.

The II-III linker of the $Ca_v2.3$ subunit is lacking the classical so called "synprint-site", which in $Ca_v2.1$ (P-/Q-type) and $Ca_v2.2$ (N-type) was shown to be responsible for the excitation secretion coupling (Mochida et al. 1996; Rettig et al. 1996) and which is responsible for synaptic vesicle endocytosis (Watanabe et al. 2010). The II-III linker of $Ca_v2.3$ however harbors a unique site located within the arginine-rich stretch, which is responsible for a novel calcium-mediated modulation of the $Ca_v2.3$ voltage-gated calcium channel (Leroy et al. 2003). This site may be involved in protein kinase C (PKC) mediated signaling (Klöckner et al. 2004), connecting $Ca_v2.3$ to muscarinic receptor activation (Mehrke et al. 1997; Meza et al. 1999; Melliti et al. 2000; Bannister et al. 2004), possibly representing the mechanism behind muscarinic enhancement of the "toxin-resistant" R-type calcium

current in hippocampal CA1 pyramidal neurons (Tai et al. 2006). The relation of this mechanism to experimentally induced epilepsy was recently summarized (Weiergräber et al. 2006, 2010; Siwek et al. 2012).

$Ca_v2.3$ contains a carboxyterminal calcium/calmodulin interaction site (Liang et al. 2003; Kamp et al. 2012a), like other voltage-gated ion channels, for example the DIII-IV linker of the cardiac sodium channel involved in action potential generation and propagation (Sarhan et al. 2012).

7.6 Interaction Partners of the Cytosolic II-III Linker of the $Ca_v2.3$

Protein interaction partners of the II-III linker of the $Ca_v2.3$ VGCC have been shown to modulate surface expression of the channel and are thought to enable binding of PKC. The amyloid-precursor-like protein APLP1 interacts with the II-III loop of the $Ca_v2.3$ VGCC increasing internalization of the channel. The small G-protein Rab5a on the other hand, which also binds to the II-III linker, modestly increasing internalization, reduces APLP1-mediated internalization of the $Ca_v2.3$ VGCC. Both interactions may represent a mechanism that maintains calcium homeostasis by regulating surface expression of the $Ca_v2.3$ VACC. Hsp70 also binds to the II-III linker of the $Ca_v2.3$ VGCC, possibly enabling phosphorylation of the channel by its known interaction partner PKC, increasing activation, as found in other VGCCs.

7.6.1 APLP1-Mediated Internalization of $Ca_v2.3$ Voltage-Gated Calcium Channels

Recently, the amyloid-precursor-like protein APLP1 was identified as a novel interaction partner of the II-III loop of the $Ca_v2.3$ VGCC, which consists of a part of the extracellular region, the transmembrane domain, and a short part of the cytosolic domain, predicted to be 6 aa in length, representing the minimum length for possible protein-protein interaction (Radhakrishnan et al. 2011b).

Amyloid precursor proteins compose a highly conserved gene family which includes APLP1 and APLP2 as well as APP, a protein crucial in Alzheimer's disease. Although various functions of these proteins have been suggested, it remains unclear whether they act as signaling receptors and/or adhesion molecules or whether their physiological function may be primarily related to their shedded soluble fragments (Jacobsen and Iverfeldt 2009). APP and APLP2 are predominantly located in intracellular compartments, whereas APLP1 is found mainly on the cell surface (Kaden et al. 2009), and is restricted to the nervous system (Slunt et al. 1994). Interestingly, synthetic peptides corresponding to the cytoplasmic domain of APLP1 and APLP2 have been shown to be phosphorylated by protein kinase C, which also

phosphorylates APP (Gandy et al. 1988; Suzuki et al. 1997; da Cruz e Silva et al. 2009). Like APP and APLP2, APLP1 also undergoes intra-membrane proteolysis (Cong et al. 2011). Furthermore, it has recently been shown that APP regulates the expression of $Ca_v1.2$ (L-type) calcium channels in striatal and hippocampal GABAergic inhibitory neurons (Yang et al. 2007, 2009).

APLP1 consists of 650 amino acids and interacts with the II-III loop of the $Ca_v2.3$ VGCC via a site between 999 and 1,899 bp, referred to here as APLP1S. Interaction of APLP1 and $Ca_v2.3$ causes an increase in internalization of $Ca_v2.3$ in stably transfected HEK 293 cells (Radhakrishnan et al. 2011b). Interestingly, the full length protein alone, and not APLP1S, which lacks part of the extracellular region, causes internalization of $Ca_v2.3$, suggesting that a signal which the extracellular region of APLP1 receives is important for endocytosis of $Ca_v2.3$. Furthermore, full length APLP1 affects inactivation kinetics of $Ca_v2.3$ VGCCs (Radhakrishnan et al. 2011b). The necessity of full length APLP1 as opposed to APLP1S, the interaction site identified in a Y2H screen in which the II-III loop of $Ca_v2.3$ was used as bait, for internalization and modulation of $Ca_v2.3$, may be based on the need for oligomerization of APLP1 via the extracellular domain, which is not uncommon for proteins of this family (Kaden et al. 2012).

APLP1 plays an important role in α_2-adrenergic receptor trafficking and may similarly act as a negative-feedback mechanism of $Ca_v2.3$ by mediating its internalization. This mechanism could represent a neuroprotective role of APLP1, reducing calcium influx into neurons, possibly activated by increased calcium influx. This is in line with findings demonstrating that expression of APLP1 mRNA is down regulated in pilocarpine-induced epileptic rats (Wang et al. 2009). Under these circumstances non-availability of APLP1 for endocytosis of $Ca_v2.3$ could lead to elevated intracellular calcium levels, possibly contributing considerably to pilocarpine-induced epilepsy and neurodegeneration. Further support of this view is given by the observation that $Ca_v2.3$ knockout mice are neuroprotected after kainate injection compared to wild type mice (Weiergräber et al. 2007), pointing to possible role of APLP1 in neurodegenerative disease.

7.6.2 Rab5A-Mediated Internalization of $Ca_v2.3$ Voltage-Gated Calcium Channels

Rab5A belongs to the Rab protein family, which comprises more than 60 proteins and can be classed as members of the small G protein superfamily. GTP-dependent Rab proteins regulate various steps of vesicular trafficking, behaving as membrane-associated molecular switches (Pochynyuk et al. 2007). Rab GTPases can associate with motor complexes, and thus, can allow for membrane association and directional movement of various vesicular cargos along the microtubule cytoskeleton (Horgan and McCaffrey 2011). Rab5A is found on the cell membrane, early endosomes and melanosomes, and is known to support the fusion of endocytotic vesicles and the

formation and transport of early endosomes (Zerial and McBride 2001). Recently it has been demonstrated that Rab5A regulates EGFR endocytosis and signaling by interacting with a protein complex consisting of TIP30, endophilin B1 and acyl-CoA synthetase long-chain family member 4, underlining its role as an endocytotic protein (Zhang et al. 2011).

Rab5A has recently been found to interact with the II-III loop of $Ca_v2.3$, modestly increasing internalization of the $Ca_v2.3$ VGCC. Intriguingly however, Rab5A reduces APLP1-mediated internalization of the channel by increasing endocytosis of APLP1 itself thus limiting the availability of APLP1 at the cell surface (Radhakrishnan et al. 2011b). These findings are in agreement with data reporting co-localization of Rab5A with APP family proteins (Marquez-Sterling et al. 1997; Kyriazis et al. 2008). One may conclude that Rab5A together with APLP1 is involved in a mechanism that maintains calcium homeostasis by regulating surface expression of the $Ca_v2.3$ VGCC.

7.6.3 Interaction of HSP-70 with $Ca_v2.3$ Voltage-Gated Calcium Channels

Heat shock 70-kDa proteins (Hsp70s) represent the most conserved family of proteins found in all organisms (Gupta 1998) and are known to be inducible by cellular stress, hyperthermia and infection (Gupta et al. 2007, 2010). Although the 13 Hsp70 isoforms account for 2 % of all proteins in stressed human cells (Zylicz and Wawrzynow 2001), they are also found in unstressed cells in which they act as chaperones (Sfatos et al. 1996; Bukau et al. 2006). In co-immunoprecipitation experiments the II-III loop of $Ca_v2.3$ was found to interact with Hsp70 (Krieger et al. 2006), which is known to interact with PKC (Newton 2003). When PKC is activated, it becomes highly sensitive to dephosphorylation. Hsp70 is capable of binding to the dephosphorylated motif and stabilizing it. PKC becomes rephosphorylated and is able to re-enter the pool of signalling-competent PKC (Gao and Newton 2002; Newton 2003).

It has been reported that $Ca_v2.2$ (N-type) $\alpha 1$ subunits are regulated by PKC dependant phosphorylation of the cytosolic linker that connects domain I and II (Zamponi et al. 1997). Similarly, $Ca_v2.3$ currents are potentiated by PKC-dependant phosphorylation at common sites shared with $Ca_v2.1$ and $Ca_v2.2$ channels but also at sites unique to $Ca_v2.3$. Examination of the effect of the PKC activator phorbol ester on $Ca_v2.3$ currents revealed that the II-III loop is an important determinant of activation, however no phosphorylation of the II-III loop could be detected therein (Krieger et al. 2006). It is assumable that PKC does not bind directly to the channel, but that Hsp70 mediates binding of PKC to the II-III loop to support phosphorylation of other regions of the channel protein to increase activation (Kamatchi et al. 2003, 2004). The interaction of Hsp70, PKC and the II-III loop of Cav2.3 has not been understood completely and it is assumable that additional proteins participate in

forming a multimeric activation complex, however involvement of Hsp70 with $Ca_v2.3$ has several possible implications for pathologies in which both proteins are involved like ischemic heart disease, diabetes and neurodegeneration.

7.7 Interaction Partners of the Carboxy-Terminal Region of $Ca_v2.3$ Voltage-Gated Calcium Channels

Specific regulation of $Ca_v2.3$ by carboxy terminal protein interaction partners plays an important role in neurotransmitter release and in presynaptic plasticity (Dietrich et al. 2003; Kamp et al. 2005). A novel calmodulin splice variant was recently shown to interact with $Ca_v2.3$, possibly modulating its gating properties and/or trafficking, linking $Ca_v2.3$ to regulation of long-term potentiation (Kamp et al. 2012a). Furthermore the G1 subunit of vacuolar ATPase, a critical protein in vesicular fusion, also binds to the C-terminus of $Ca_v2.3$. Inhibition of V-ATPase attenuates the $NiCl_2$ mediated increase of the R-type-dependent b-wave measured in electroretinograms and reduces $Ca_v2.3$ peak currents indicating a role for $Ca_v2.3$ in exocytosis and thus neurotransmitter release.

7.7.1 Interaction of $Ca_v2.3$ Voltage-Gated Calcium Channels and Calmodulin

Because calcium is an important second messenger and is involved in major cellular processes, such as exocytosis and induction of apoptosis, regulation of calcium influx and thus of calcium homeostasis is critical for the cell. Calmodulin (CaM) is a central molecule in cellular calcium regulation acting on over 300 different target proteins (Findeisen and Minor 2010). Structurally, CaM is composed of two independent lobes (C- and N-lobe) each with two EF-hands as calcium-binding motifs.

CaM regulates VGCCs by interacting with the IQ-domain, in the carboxyterminus (Zühlke et al. 2000). Generally, CaM has two different modulatory effects on VGCCs: (i) calcium-dependent facilitation (CDF) and (ii) calcium-dependent inactivation (CDI). CDI of Ca_v1 channels is mediated by the C-lobe of CaM whereas the N-lobe of CaM drives CDI in the Ca_v2 subfamily (Peterson et al. 1999; DeMaria et al. 2001; Liang et al. 2003). It has been suggested that the differences in lobe-specific function of CaM between Ca_v1 and Ca_v2 subfamilies are due to differences in binding orientation of Ca_v1 and Ca_v2 calcium channels (Findeisen and Minor 2010).

Recently, a novel splice variant of CaM-2 (CaM-2-ext) with a 46 nucleotide- long insertion retained from a 5666 nucleotide-long intron between exon 1 and 2 of the

classic calmodulin-2 has been found in two human cell lines and was identified as an interaction partner of the carboxyterminus of $Ca_v2.3$ by yeast-two-hybrid screening and co-immunoprecipitation (Kamp et al. 2012a). CaM-2-ext significantly decreases $Ca_v2.3$ peak current density, which may be caused by modulation of $Ca_v2.3$ channel gating properties or impairment of its trafficking (Kamp et al. 2012a). The physiological and pathophysiological significance of CaM-2-ext as well as its expression pattern must be further investigated in future studies.

Modulation of VGCCs, particularly of $Ca_v2.3$ by CaM appears to be important for presynaptic calcium regulation. It is conceivable that CDI of $Ca_v2.3$ relies on the global presynaptic calcium concentration sensed by CaM, indicating an important role of CaM as a sensitive calcium concentration sensor (Liang et al. 2003). Furthermore, there is strong evidence that both $Ca_v2.3$ and CaM, are involved in the induction of presynaptic long-term potentiation (LTP) in certain synapses such as in mossy fibers and cerebellar Purkinje cell terminals (Dietrich et al. 2003; Breustedt et al. 2003; Myoga and Regehr 2011). Calcium entering the cell through $Ca_v2.3$ binds to CaM, which may activate adenylyl cyclases and subsequently protein kinase A (PKA) leading to induction of presynaptic LTP (Kamp et al. 2005, 2012). LTP also involves PKC activation, which in turn modulates $Ca_v2.3$ by increasing presynaptic calcium influx through $Ca_v2.3$ (Stea et al. 1995; Klöckner et al. 2004).

7.7.2 Interaction of $Ca_v2.3$ Voltage-Gated Calcium Channels with Vacuolar ATPase

Recently, the G1 subunit of the vacuolar ATPase (V-ATPase) was identified as a novel interaction partner of the carboxyterminus of $Ca_v2.3$ voltage-gated calcium channels. V-ATPases are highly conserved multi-enzyme complexes, which consist of a peripheral, catalytic (V1) and a membrane-integrated sector (V0) (Nelson and Harvey 1999; Nishi and Forgac 2002). The G1 subunit is part of a peripheral stalk connecting both sectors and is involved in the regulation of the multi-enzyme complexes' stability (Charsky et al. 2000). As V-ATPases pump protons under ATP-hydrolysis through cellular membranes they are involved in various cellular processes such as vesicle acidification, protein processing, and their trafficking and targeting (Palokangas et al. 1998; Gruber et al. 2001; Schoonderwoert and Martens 2001).

Recently, the G1 subunit of the V-ATPase was identified as a novel interaction partner of the full length $Ca_v2.3$ C-terminus by yeast-2-hybrid screening (Radhakrishnan et al. 2011a). This interaction was confirmed by FLAG immunoprecipitation in 293 T cells. Similarly, Gao and Hosey identified the homolog G2 subunit of V-ATPase as an interaction partner of the L-type calcium channel $Ca_v1.2$ by similar methods and using a GST-pull down assay (Gao and Hosey 2000). Nevertheless, the physiological significance of the interaction between the V-ATPase and VGCCs remains unclear. The V-ATPase inhibitor bafilomycin

A1 reduces $Ca_v2.3$ peak currents and attenuates the $NiCl_2$ mediated increase of the R-type-dependent b-wave measured in electroretinograms (Radhakrishnan et al. 2011a). Whether bafilomycin affects the interaction between $Ca_v2.3$ and the V-ATPase however, is uncertain. More likely, trafficking of VGCCs to the plasma membrane is affected by the V-ATPase antagonist leading to reduced calcium channel currents. This interpretation is in line with the previous results from Gao and Hosey who observed disturbed trafficking of $Ca_v1.2$ calcium channels to the plasma membrane and their intracellular accumulation after treatment with the V-ATPase inhibitor folimycin (Gao et al. 2001).

Furthermore, interaction of VGCCs with V-ATPase could be critical in the mechanism of exocytosis: the V0 sector of V-ATPase was suggested to act as a fusion pore during exocytosis (Morel et al. 2001; El Far and Seagar 2011). The V0 sector is composed of a ring of homolog subunits enriched in the presynaptic membrane (Taubenblatt et al. 1999; Morel et al. 2001). It interacts with several proteins of the exocytotic machinery such as VAMP, syntaxin and synaptobrevin (Galli et al. 1996; Shiff et al. 1996; Morel et al. 2003) and is calcium-sensitive and permeable to acetylcholine. The V0-proteolipid rings have shown to be involved in membrane fusion in yeast vacuoles (Peters et al. 2001; Bayer et al. 2003). Thereby, two proteolipid rings in both membranes dimerize in a "head-to-head" position forming a channel. Membrane proteolipids can invade the V0-proteolipid ring connecting both membranes promoted by lateral separation of the V0 proteolipid ring subunits (Peters et al. 2001; Bayer et al. 2003). A similar mechanism was suggested to occur during exocytosis, however more data is needed on this subject. During docking of the synaptic vesicle to the active zone of neurotransmitter release, the interaction between VGCCs and V-ATPase may help organize of the synaptosome and possibly destabilize the V-ATPase holoenzyme leading to dissociation of the V1 sector from the V0 sector. It is conceivable that formation of a loose and—after rising of presynaptic calcium—tight SNARE complex positions the V0 sector in the vesicle and the plasma membrane, rendering dimerization of V0 sectors and subsequent neurotransmitter release highly dependent on direct interaction with VGCCs, however, experimental data in support of this hypothetical model has yet to be provided.

7.8 Future Outlook: Role of $Ca_v2.3$ Channels and Their Interaction Partners in Cardiac Activity

L-type channels are not the only VGCCs in cardio myocytes: T-type and more recently R-type channels have been identified in the myocardium, however the influence of $Ca_v2.3$ VGCCs on cardiac activity is still being debated. $Ca_v2.3$ deficient mice display arrhythmic patterns like uncoordinated atrial activation, second degree atrioventricular block type II (Mobitz type II) and QRS-dysmorphology. The exact mechanism of action has yet to be elucidated, however a role of $Ca_v2.3$ in

successive activation of voltage-gated calcium channels has been suggested. Thus, further studies of the functional role of $Ca_v2.3$ and its modulation by interaction partners in cardiac activity could be of great physiological and pathophysiological importance.

The VGCCs investigated in greatest detail in the myocardium are the L-type channels. Of particular importance among these, is the $Ca_v1.2$ channel as the main contributor of excitation-contraction coupling (Wang et al. 2004; Brette et al. 2006). Upon cardiomyocyte depolarization, L-type calcium channels open allowing influx of calcium ions which activates ryanodine receptors (RyR)—particularly RyR2—, resulting in a release of calcium ions from the sarcoplasmic reticulum into the cytosol, i.e. calcium-induced calcium release (Valdeolmillos et al. 1989). The importance of $Ca_v1.2$ in the myocardium is underlined by the non-viability of mice lacking the channel, which die before day 14.5 p.c., i.e. 1 day after the embryonic heart starts beating (Seisenberger et al. 2000).

Nevertheless, $Ca_v1.2$ channels are not the only VGCCs in cardiomyocytes. Other L-type channels ($Ca_v1.3$) (Mangoni et al. 2003; Marger et al. 2011; Qu et al. 2011), T-type channels ($Ca_v3.1$ and 3.2) (Cribbs 2010; Ono and Iijima 2010; Marger et al. 2011), and more recently R-type channels ($Ca_v2.3$) (Mitchell et al. 2002; Lu et al. 2004; Weiergräber et al. 2005; Murakami et al. 2007) and Galetin, Schneider et al., unpublished) have been identified in the myocardium. The role of $Ca_v1.3$ channels in cardiac activity is generally well accepted and is reported to play a compensatory role after $Ca_v1.2$ ablation (Xu et al. 2003). The function of $Ca_v2.3$VGCCs on the other hand, is still being debated.

Significant evidence pointing towards a non-negligible role of $Ca_v2.3$ channels in cardiac pacemaking is continuously being raised. Weiergräber et al. and Mitchell et al. detected both $Ca_v2.3$ channel expression at both mRNA and protein in rat atrial and ventricular myocytes (Weiergräber et al. 2000; Mitchell et al. 2002). Shortly thereafter, a significantly increased coefficient of variation in heart rate was found in isolated embryonic hearts of $Ca_v2.3$ deficient mice, reflecting increased variability of heart rate and an irregular beating pattern (Lu et al. 2004). In hearts of adult $Ca_v2.3$ deficient mice, telemetric ECG recording also revealed arrhythmic patterns, including ECG dysmorphology, uncoordinated atrial activation (partially non-transducted), second degree atrioventricular block type II (Mobitz type II) and QRS-dysmorphology (Weiergräber et al. 2005). Taken together, these findings point toward an important role of $Ca_v2.3$ in sustaining a regular heart beat, due to their expression in pacemaker cells, both in embryonic and adult hearts.

Despite all the previously-mentioned data, some doubts still exist as to whether $Ca_v2.3$ truly contributes to cardiac pacemaking in the myocardium or only via the autonomic nervous system. In effect, knockout animals not only display pacemaking disturbances, but also altered autonomic nervous system control after ablation of $Ca_v2.3$ (Weiergräber et al. 2005). Modified sympathetic regulation of cardiac activity is found in mice lacking Ca_v2 subfamily channels $Ca_v2.3$ and $Ca_v2.2$ (Murakami et al. 2007). In addition, expression of $Ca_v2.3$ in rat intra-cardiac neurons (although only at low levels of 7 %) has been proven (Jeong and Wurster 1997). These doubts are additionally exacerbated by difficulties in detecting $Ca_v2.3$ protein in mouse

heart microsomes so far. However, using the isolated perfused heart experimental set up (Langendorff), similar arrhythmic patterns could be recorded in spontaneously beating hearts extracted from $Ca_v2.3$-deficient mice (Tevoufouet and Schneider, unpublished). Using the Langendorff method, significantly increased heart rates were recorded from isolated perfused hearts of $Ca_v2.3$-deficient mice (Tevoufouet and Schneider, unpublished), an outcome observed in telemetric ECG recordings of $Ca_v2.3$-deficient mice (Weiergräber et al. 2005). However, in embryonic isolated hearts of $Ca_v2.3$-deficient mice, heart rate was found to be reduced

Altogether, these facts suggest that the ablation of $Ca_v2.3$ channels causes abnormalities in cardiac activity, which cannot be fully compensated by upregulation of $Ca_v3.1$ channels (Weiergräber et al. 2005), thus confirming a significant role of $Ca_v2.3$ in pacemaking of cardiac activity. The exact mechanism of action has yet to be elucidated, however a role of $Ca_v2.3$ in successive activation of VGCCs (Lakatta et al. 2010) has been suggested: after activation of T-type channels, activation of $Ca_v2.3$ could be required to achieve the potential necessary for activation of L-type calcium channels (Galetin, Schneider et al., unpublished). Thus, further studies of the functional role of $Ca_v2.3$ and its interaction partners in cardiac activity could be of great physiologic and pathophysiologic importance.

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Chapter 8
Voltage-Gated Calcium Channel Signaling to the Nucleus

Michel Bellis, Thierry Cens, Pierre Charnet, and Matthieu Rousset

Abstract Excitation-transcription coupling makes use of cellular excitability to produce intracellular signals to the nucleus to control activity-dependent gene expression. Voltage-gated calcium channels are presented here as a signaling platform able to redirect multiple signaling pathways toward the nucleus. Whilst several Ca_V subunits are implicated in excitation-transcription coupling, each type of Ca_V nevertheless possesses its own proteome and microenvironment able to promote individualized signaling pathways. L-type calcium channels have structural determinants that favor the initiation of MAPK and CamK pathways for example, but P/Q and N-type channels, in close proximity to the endoplasmic reticulum, promote calcium-induced calcium release-dependent mechanisms. Furthermore, auxiliary $Ca_V\beta 4$ subunits or truncated C-termini of $Ca_V1.2$ and $Ca_V2.1$ channels can be targeted to the nucleus and become direct messengers involved in the regulation of gene expression. These later discoveries suggest that novel pathways must be inserted in the global description of excitation-transcription coupling and give new clues to the understanding of calcium channelopathies with interesting physiopathological perspectives.

Keywords Voltage gated calcium channels • Transcription • Nucleus • Ataxia • Epilepsy

8.1 Introduction

The nervous system undergoes a constant maturation induced by diverse types of cognitive, motor, sensory or accidental experiences. These experiences produce external signals which are integrated at the plasma membrane of neuronal cells

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by numerous proteins responsible for signal reception and transduction. These transduction pathways induce various signaling cascades that regulate the activity of target proteins by direct post-translational modifications, and/or by modulation of their synthesis and degradation rates. A key pathway is the communication between these plasma membrane receptors and the nucleus, where the integration of external signals leads to the remodeling of the gene expression that promotes long-lasting modifications of neuronal activity and morphology. Here, our aim is to review the roles of the voltage-gated calcium channels (VGCCs) in such process as so-called excitation-transcription coupling (ETC), whereby neuronal depolarization due to opening of VGCCs activates specific activity-regulated transcription programs. ETC make reference to the well-described excitation-contraction coupling and excitation-secretion coupling processes, in which these VGCCs have central roles in linking electrical activity to muscle contraction (Bénitah et al. 2002) and synaptic vesicle release (Wojcik and Brose 2007; Eggermann et al. 2012), respectively.

8.2 Routes from Membrane to Nucleus

Communication between pre- and post-synapses and nucleus is well-established and pertain to two types of mechanisms (Saha and Dudek 2008; Fainzilber et al. 2011). One mechanism is characterized by a rapid transfer of external information through the axon or the dendritic tree that is ended by a somatic rise of the calcium concentration. This transfer is carried out by electrical activity of the neuron which *in fine* activate VGCCs located at the somatic compartment, or by a regenerative calcium wave that culminates in a large increase of the calcium concentration in the soma (Verkhratsky and Shmigol 1996; Rose and Konnerth 2001). The somatic calcium rise activates different types of calcium-sensitive transcription factors or calcium-sensitive transcriptional regulators that translocate into the nucleus to reach their targets. This "fast calcium track" seems to be implicated in general programs of activity-dependent gene expression such as expression of immediate early genes (IEG) involved in the synaptogenesis and neuronal plasticity processes (Alberini 2009; Barco and Marie 2011; Okuno 2011; Karpova et al. 2012; Middei et al. 2012).

Another mechanism which presents slower kinetics relies upon a physical translocation of pre or post-synaptically localized proteins to the nucleus. Usually activity-dependent post-translational modifications produce protein uncoupling from a transmembrane complex or their truncation, leading to the release of a soluble intracellular domain (ICD). In both cases, nuclear translocation occurs and the translocated functional protein regulates gene expression directly by acting as a transcription factor, or indirectly by interacting and regulating transcription factors. This "slow protein track" has been reported for several receptors such as APP, erbB4, Neuroligin or Notch, and has been proposed to be responsible for more specific responses of activity-controlled transcription (Jordan and Kreutz 2009; Ch'ng and Martin 2011; Ch'ng et al. 2012).

VGCCs are well-known activators of the fast track communication pathway, and, as new evidence demonstrates, VGCCs are also implicated in slow protein track

pathways in which VGCC subunits, or their ICDs, translocate from the plasma membrane to the nucleus. Therefore, it is tempting to speculate that VGCCs form nuclear signaling platforms able to drive different activity-regulated gene networks using specific signaling cascades, dependent on the stimulation patterns and the channel's environment (Hardingham et al. 1999; Mermelstein et al. 2001; Wu et al. 2001a).

8.3 Studies Implicating VGCCs in Fast Excitation-Transcription Coupling

Fast track gene regulation converges on a few transcription factors, such as Mef2, SRF or CREB (cAMP response element binding protein), the latter which represents the prototypical activity-regulated transcription factor. CREB contains a C-terminal DNA binding domain that recognizes the CRE (cAMP Response Element) site, and a transactivation domain. The transactivation domain contains Q1 and Q2, two glutamine-rich domains, able to interact with the transcription machinery, and a kinase-inducible domain that encloses multiple phosphorylation sites. The phosphorylation state of the kinase-inducible domain determines its ability to bind to the KIX domain of the co-factor CBP or p300 necessary to initiate transcription. In particular, the phosphorylation of serine 133 targeted by different types of kinases, including CamK, the cAMP-dependent protein kinase (PKA) or the mitogen/stress-activated protein kinase (MAPK), represents a key step of CREB activation (Dolmetsch 2003; Barco and Marie 2011; Sakamoto et al. 2011). CREB, which has been implicated, in particular, in synaptic plasticity and intrinsic plasticity, regulates the transcription of thousands of genes including immediate early genes (IEGs) like *c-fos* or *bdnf* (Sakamoto et al. 2011; Middei et al. 2012). Usually the phosphorylation state of the serine 133 of CREB and the induction of *c-fos* expression are regarded as classical markers of activity-dependent transcription. L-type, P/Q type and N-type calcium currents have all been reported to be able to activate CREB and to induce gene expression (Sutton et al. 1999; Brosenitsch and Katz 2001; Zhao et al. 2007; Wheeler et al. 2012). This coupling between VGCCs and CREB seems to be a general neuronal process as it occurs in a large diversity of neurons, from central nervous system (hippocampal formation, striatum, cortex and cerebellum), peripheral nervous system (sensory neurons) or endocrine system. However, this coupling can involve different pathways, using either propagating electrical waves or calcium regenerative waves.

8.3.1 Fast Excitation-Transcription Coupling Through Electrical Propagating Waves

Amongst the most studied properties of neurons prone to ECT are the forms of synaptic plasticity which allow the adaptation of the synaptic strength to the level of repetitive stimulation. For example, long-term potentiation and long-term

depression, which seem to be both regulated by AMPA receptor trafficking (Boehm et al. 2006; Bramham et al. 2008), are sensitive to transcription inhibitors (Squire and Barondes 1970; Abraham et al. 1991). Several lines of evidences suggest that activity-induced transcription must take place within a few minutes, if not seconds, after the stimulation (Saha and Dudek 2008). Actually, it has been demonstrated that many IEGs are induced less than 2 min after stimulation (Guzowski et al. 1999; Bottai et al. 2002; Pevzner et al. 2012). Biochemical and morphological constraints (low speed of protein transport and long distances from the nucleus) mean that membrane depolarization is one of the best ways to translate synaptic excitation to the somatic area within appropriate timeframes. As L-type calcium channels are mainly found in the somatic compartment and the proximal part of the dendritic tree, they are ideally placed to be key transducers of ECT. In fact, L-type calcium channels are implicated in CREB phosphorylation at serine 133 and in activity-dependent *c-fos* gene expression in different neuronal types and following diverse stimulations (Greenberg et al. 1986; Morgan and Curran 1986; Murphy et al. 1991; Hardingham et al. 1997; Liu and Graybiel 1996; Rajadhyaksha et al. 1999; Tolón et al. 2000; Dolmetsch et al. 2001; Macías et al. 2001). L-type calcium current induces activation of CamKII/IV less than 60 s after channel opening. CamK activation is an essential step of *c-fos* gene induction as it phosphorylates CREB at serine 133 (Bading et al. 1993). It has also been shown that N-type and P/Q type VGCCs are also able to phosphorylate CREB and activate *c-fos* expression (Brosenitsch and Katz 2001; Zhao et al. 2007; Wheeler et al. 2012).

8.3.2 Fast Excitation-Transcription Coupling Through Calcium Propagating Waves

An alternative route involves a synaptic calcium rise which triggers a calcium-induced-calcium-release mechanism along dendrites to produce a regenerative calcium wave that culminates in a large increase of the calcium concentration in the soma. The initial calcium increase arises from VGCC opening by membrane depolarization or by ligand binding to ionotropic receptors (for example, NMDA or AMPA receptors) or to metabotropic receptors that produces, respectively, calcium influx or calcium release from reticulum endoplasmic. Sutton et al. have shown that *syntaxin1a* gene transcription is controlled by such calcium-induced-calcium release mechanism (Sutton et al. 1999). Calcium entry through P/Q type calcium channels was able to trigger expression of syntaxin1a in $Ca_V2.1$-overexpressing HEK-293 cells and P/Q type calcium current block by ω-agatoxin IVA in cerebellar granule cells prevented *syntaxin1a* mRNA production. The pathway recruited was calcium dependent and required the integrity of the reticulum endoplasmic since xestospongin C, a specific inhibitor of IP3 receptors, and dantrolene, an inhibitor of store-operated calcium release, both blocked *syntaxin1a* gene transcription. It was also observed that P/Q type calcium current increased CREB phosphorylation and that gene expression required the activation of CamK, MAPK kinase and PKA (Sutton et al. 1999).

In hippocampal neurons, L-type calcium channels are also able to target CREB directly to the nucleus by a calcium-induced-calcium-release mechanism. Using an elegant approach, the group of Bading has shown that calcium influx through VGCC triggers a calcium rise in the nucleus, which induces phosphorylation of CREB on serine 133 even in conditions where the nuclear pore complex had been previously blocked by injection of wheat germ agglutinin (Hardingham et al. 2001); treatment of wheat germ agglutinin is considered to completely prevent nucleocytoplasmic shuttling of proteins. Moreover, this group had previously demonstrated that chelation of nuclear calcium was sufficient to inhibit CREB phosphorylation and *c-fos* expression (Hardingham et al. 1997; Chawla et al. 1998). These experiments demonstrated the propagation of a calcium wave between the L-type channels and the nucleus which was able to trigger activity-dependent gene expression without any physical translocation of proteins into the nucleus. Moreover, these experiments have also shown that the nucleus contains all the elements required to activate CREB-dependent transcription and can operate independently under the occurrence of a nuclear calcium rise.

CREB is not the only transcription factor that can be activated by a regenerative calcium wave; other transcription factors contain calcium binding sites. DREAM, which is a transcription factor able to bind DNA on a DRE site and belongs to the recoverin calcium binding protein family, has four EF hands, of which three are functional. DREAM activity is under the control of nuclear calcium concentration. In low calcium concentrations DREAM forms a tetramer which is able to bind DNA and act as a repressor by preventing transcription initiation. When nuclear calcium concentration increases, the tetramer of DREAM is split into dimers which is unable to bind DNA and thereby releases transcription (Carrión et al. 1999; Savignac et al. 2005, 2007). Finally a regenerative calcium wave that culminates into the nucleus is clearly able to regulate DREAM activity. Leclerc et al. have demonstrated that *GnRH* gene expression in GT1-7 cells is under the control of both L-type VGCC activity and DREAM transcription factor, leaving open the possibility of a direct coupling between these components via a calcium-induced-calcium-release mechanism (Leclerc and Boockfor 2007).

8.3.3 Are There VGCC Specialized for Fast Excitation-Transcription Coupling?

L-type channels were the first discovered and best studied VGCC involved in ECT. Their somatodendritic localization and the lack of other critical neuronal functions led to the consideration that they were optimal for ETC. Other VGCC could be also implicated, but in a less direct way by contributing to the bulk cytoplasmic calcium pool that activates gene transcription in a nonspecific fashion. This idea was strengthened by the observation that in cortical and sensory neurons, despite a minor role in depolarization-induced increases in calcium, L-type channels play a

major role in activity-regulated gene expression (Mintz et al. 1991; Murphy et al. 1991; Brosenitsch et al. 1998; Dolmetsch et al. 2001; Zhao et al. 2007; Wheeler et al. 2012). However, the experimental conditions used to analyze L-type dependent ETC usually rely on the application of high extracellular potassium concentration, which induce a chronic membrane depolarization similar in amplitude to the depolarization observed during excitatory postsynaptic potentials. Such conditions are non-physiological and lead to a systematic inactivation of non-L-type VGCCs (Nowycky et al. 1985; Fox et al. 1987; Dolmetsch et al. 2001; Liu et al. 2003). Moreover, 40 mM KCl stimulation, which is the most commonly used KCl concentration, corresponds to an activation plateau maintained at −20 mV, meaning that Ca_V1 channels contribute to more than 75 % of the calcium current; this is mainly due to different biophysical properties between Ca_V1 and Ca_V2, as the latter are not activated at such potentials (Mermelstein et al. 2000; Wheeler et al. 2012). Together, this tends to underestimate the participation of Ca_V2 channels to ECT. Consequently, Ca_V2 channel contribution must be evaluated in presence of higher KCl concentrations (>60 mM) or especially using more physiological stimulations.

In primary sensory neurons, Brosenitsch and co-workers have shown that patterned electrical field stimulation at 5 Hz induces expression of the tyrosine hydroxylase *Th* gene (Brosenitsch and Katz 2001). In this case, ECT was neither sensitive to nimodipine nor CamK or MAPK inhibitors, but was sensitive to ω-conotoxin GVIA, a specific N-type VGCC blocker, and to protein kinase PKA/PKC inhibitors. Interestingly, in superior cervical ganglion neurons, 10 Hz electrical field stimulation induced an ETC that was completely blocked either by L-type blocker (nimodipine) or N-type blocker (ω-conotoxin GVIA) (Zhao et al. 2007). It is worth noting that, in the same preparation, KCl-induced-ETC is only sensitive to nimodipine; Increasing the frequency of stimulation to 50 Hz makes the ETC sensitive only to L-type blockers (Zhao et al. 2007). These results suggest that a large range of frequencies are able to induce ETC mediated by different VGCCs. Moreover, L-type VGCCs target CREB only after synaptic potentials while they are opened either by synaptic potentials or back propagating action potentials (Regehr and Tank 1992; Mermelstein et al. 2000; but see Dudek and Fields 2002). ETC is thus not an ON/OFF mechanism coupled to the opening of the channel but requires an adequate stimulation. Finally two different types of L-type channels present different abilities to target CREB depending on the amplitude of the stimulation. Using low potassium concentration, $Ca_V1.3$ is more effective than $Ca_V1.2$ in inducing CREB phosphorylation; however, increasing the extracellular potassium concentration make $Ca_V1.2$ more effective than $Ca_V1.3$ (Zhang et al. 2006). Together, these data suggest that neurons trigger different fast track ETCs corresponding to different types of electrical activity using specific VGCC and downstream signaling.

Overall, L-type channels seem to be more efficient in signaling CREB than Ca_V2 channels. It has been shown that at equal calcium influx through Ca_V1 and Ca_V2 channels, CREB phosphorylation level is 10 fold greater after Ca_V1-channels

activation (Wheeler et al. 2012). Moreover, in sympathetic neurons, ETC induced by electrical field stimulation at 10 Hz involves cooperatively between L and N-type VGCC; indeed, the ETC is completely blocked by L-type blockers or by N-type blockers, meaning that calcium influx through both channels is required (Zhao et al. 2007). It has been proposed recently that during an ECT episode, CamK activation requires a clustering step in close vicinity to the Ca_V1 VGCC, independently of the calcium source. Therefore calcium influx through Ca_V2 channels induces a translocation of CamKII near or in the macromolecular complex of Ca_V1 channels (Hudmon et al. 2005a; Wheeler et al. 2008, 2012). In this regard, the privileged ability of L-type channels for ETC seems to come from the specialization of the nanodomain around the mouth of the channel. The L-type channel-nanodomain regroups at the submicron scale the key signaling proteins required for the onset of ETC. Calmodulin and CamKII have been already shown to interact with $Ca_V1.2$ (Zühlke and Reuter 1998; Mori et al. 2004; Hudmon et al. 2005b; Xiong et al. 2005; Grueter et al. 2006, 2008; Fallon et al. 2009; Abiria and Colbran 2010). Moreover, $Ca_V1.2$ and $Ca_V1.3$ channels possess PDZ motifs on their C-terminal extremity that usually allows transmembrane proteins to bind to the cytoskeleton, thus contributing to the submembranous architecture organization. It has been shown that these domains are critical to L-type-dependent ETC (Weick et al. 2003; Zhang et al. 2005) and allow L-type channels to bind to scaffolding protein shank or neuronal-interleukin-16 (Kurschner and Yuzaki 1999; Zhang et al. 2005). These results suggest that PDZ sequences specifically found on L-type channels organize a macromolecular complex dedicated to ETC. Similarly, the Ca_V2 nanodomain seems to be buffered by endoplasmic reticulum and mitochondria (Akita and Kuba 2000; Wheeler et al. 2012). The functional consequences are double: first, a large part of Ca_V2 calcium influx is collected by the endoplasmic reticulum and mitochondria which limit the size of calcium nanodomain at the mouth of these channels; second, the close proximity of endoplasmic reticulum favors the activation of ryanodine receptors by Ca_V2-dependent calcium influx which, in turn, triggers a calcium-induced calcium release, phenomenon that can leads to the CREB phosphorylation (Sutton et al. 1999). Again, this specialized organization around the Ca_V2 channel mouth probably requires protein-protein interactions. Recent studies on the Ca_V2 proteome that point out multiple interactions with adaptors and cytoskeleton proteins could be a valuable source of data to study the Ca_V2-reticulum endoplasmic interaction (Muller et al. 2010).

Each class of VGCC displays specific biophysical properties which allow neurons to respond to diverse electrical stimulations, such as synaptic potentials and action potentials, for specific ECT processes. The partition of fast track ETC into use of electrical waves or calcium waves has origins in the nanodomain of each VGCC. Due to their close proximity with the endoplasmic reticulum, the Ca_V2 channel family is prone to calcium-induced-calcium-release mechanisms that culminate with a calcium rise in somatic space or directly within the nucleus, whilst the Ca_V1 family initiates CREB activation directly in response to electrical activity.

8.4 The Slow Protein Track: Long-Distance Physical Translocation of Ca_V-Bound Signaling Proteins to the Nucleus

As we have already mentioned, CREB can be activated by others kinases including MAPK and PKA. Cortical or hippocampal neurons stimulated by KCl induce a robust CREB phosphorylation lasting more than 1 h. It appears, in fact, that CREB activation follows two overlapping phases. The first phase is triggered within 1 min and lasts around 20 min, whereas the second phase starts 15 min after the stimulation and lasts for more than 1 h. Although the first phase seems not to be dependent on L-type channels and can use Ca_V2 channels (Murphy et al. 1991; Frödin and Gammeltoft 1999; Pearson et al. 2001), it appears that the second phase is clearly dependent on L-type channels, as nimodipine blocks its activation (Dolmetsch et al. 2001). The first phase, sensitive to KN-93, which is a potent inhibitor of CamK pathway, corresponds to the fast-track gene regulation pathway that we have already described. The second phase is sensitive to a dominant-negative form of Ras, to the MAPK kinase inhibitor PD98059 and proceeds with a sustained phosphorylation of ERK, indicating the activation of this kinase. Together, these data indicate that the second phase of CREB phosphorylation is performed by the MAPK cascades that have been extensively described elsewhere (Pearson et al. 2001). Using an elegant approach, Dolmetsch et al. demonstrated that the calcium sensor involved in MAPK activation is L-type channel bound calmodulin (Dolmetsch et al. 2001). The principle of the "functional knock in" technique they developed is to replace the endogenous channels by recombinant channels that have been mutated in order to investigate the role of a given functional site. Recombinant channels were also mutated at amino acids necessary for dihydropyridine block; using this approach, it was shown that the IQ domain, which allows the binding of calcium associated calmodulin to the channel, is necessary for ERK phosphorylation and the late phase of CREB activation (Dolmetsch et al. 2001). These results imply that the activation of the MAPK pathway occurred in the nanodomain of L-type channels.

Accordingly, it appears that activation of L-type channels is able to trigger two different signaling cascades from its macromolecular complex which both converge on the phosphorylation of CREB at serine 133. The differences between these two pathways lie on the stimulation strength and the kinetics of the cascades. The MAPK pathway requires a strong depolarization e.g. 90 mM KCl application, but is unresponsive to 20 mM KCl, which produces a smaller calcium rise; the key point is the lag time before CREB phosphorylation; whereas CamK pathway activates CREB almost immediately, the MAPK pathway requires more than 15 min (Wu et al.2001b). Why is there such a delay? Dolmetsch et al. (2001) demonstrated that the kinase ERK is activated less than 1 min after stimulation, suggesting that the period before CREB phosphorylation corresponds to the length required for

the activated ERK to reach the nucleus. Alternatively, activated ERK could act by targeting other kinases like Rsk1/2 or Msk, which in turn will directly phosphorylate CREB. In this case, it is possible that an extended time is needed for Rsk/Msk activation or for trafficking from the phosphorylated Rsk/Msk to CREB (Frödin and Gammeltoft 1999).

Another example of slow ETC mediated by uncoupling of Ca_V partners is illustrated by the NFAT signaling pathway. NFAT transcription factor represents five proteins, NFATc1-4 and NFAT5, which, with the exception of NFAT5, respond to cytoplasmic calcium rise by a dephosphorylation step mediated by calcineurin, a calcium-calmodulin activated phosphatase. NFATc1-4 are all highly expressed in the peripheral and central nervous system. The structure of NFAT is composed of two parts: an N-terminal regulatory domain called the NFAT homology region (NHR) which contains two calcineurin binding sites, the calcium-independent PXIXIT site, and the calcium-dependent LXVP site, and a DNA binding domain similar to the Rel/NFkappaB DNA binding domain. The NHR domain contains a nuclear localization sequence (NLS) controlled by the NFAT phosphorylation state. During basal conditions, cytoplasmic NFAT is highly phosphorylated at the NHR region, which probably masks the NLS. When the calcium concentration rises, calcium-calmodulin binds and activates calcineurin which, in turn, dephosphorylates NFAT allowing nuclear translocation of the transcription factor (Moore and Goldberg 2011).

In peripheral and central neurons, NFATc4 and NFATc3 are translocated to the nucleus after 3 min of 5 Hz stimulation or 90 mM KCl application; spontaneous synaptic activity of the neuron is also able to induce a NMDA-sensitive nuclear translocation (Graef et al. 1999; Ulrich et al. 2012). This translocation is associated with the transcription of specific genes like *IP3R1* gene and is sensitive to the calcineurin blockers FK506 and cyclosporine. Moreover, NFATc4/c3 nuclear translocation and *IP3R1* gene expression are increased by the L-type VGCC agonist BayK8644 and abolished by L-type antagonist nifedipine, but not by Ca_V2 blockers (Genazzani et al. 1999; Graef et al. 1999). It is worth noting that whereas both NFATc isoforms are able to enter the nucleus under strong depolarization, only NFATc3 is implicated in a nucleocytoplasmic shuttling under milder stimulation e.g. chronic membrane depolarization in response to 20 mM KCl. These dissimilar properties are explained by a differential sensitivity of each NFATc to GSK3β kinase; for example, GSK3β represses nuclear localization of NFATc4 by phosphorylating the C-terminal part of the NHR domain (Graef et al. 1999; Ulrich et al. 2012).

Oliveria et al. (2007) have shown using biochemical and FRET approaches that $Ca_V1.2$ channels interact with A-kinase anchoring protein 79/150 (AKAP79/150), and that AKAP79/150 binds to calcineurin. This macromolecular $Ca_V1.2$-calmodulin-AKAP79/150-calcineurin complex is required for NFAT signaling and AKAP79/150 knock-down abolishes KCl-induced NFATc4 nuclear translocation (Oliveria et al. 2007). The differential sensitivity to BAPTA and EGTA confirmed findings that calcineurin activation occurs in the nanodomain of $Ca_V1.2$. The most

likely scheme is that calcium influx through $Ca_V1.2$ channels binds to the IQ bound calmodulin that activates calcineurin, which, in turn, binds to NFATc. As a single calcineurin is probably unable to bind at the same time to both the PXIXIT site of AKAP79/150 and to NFATc, we can speculate that an uncoupling of calcineurin from the $Ca_V1.2$-calmodulin-AKAP79/150 complex occurs. NFATc starts to become located in the nucleus 15 min after the stimulation, and, in parallel, *IP3R1* expression become barely visible 1 h after the KCl stimulation (Genazzani et al. 1999; Graef et al. 1999). NFATc-dependent ETC is a slow process arising probably from NFAT4c nucleocytoplasmic shuttling.

Two additional nucleocytoplasmic shuttling processes that may be implicated in an ETC in neurons should also be mentioned. We have already described that DREAM is a transcriptional repressor when it is localized in the nucleus. However, DREAM is also present in the cytoplasm, mainly in the perinuclear area (Pruunsild and Timmusk 2012), and can translocate in the nucleus after sumoylation (Palczewska et al. 2011). In cardiomyocytes, CamKII regulates the DREAM nucleocytoplasmic ratio and, in parallel, DREAM regulates L-type channel expression by binding on the DRE site of $Ca_V1.2$ promoter (Ronkainen et al. 2011). Moreover, a clear correlation between the level of expression of CamKII and the expression of $Ca_V1.2$ channel has been shown (Xu et al. 2010; Ronkainen et al. 2011). An interesting point of this study is the modification of the calcium influx through L-type channels by BayK 8644 application, which favors the nuclear localization of DREAM (Ronkainen et al. 2011). Together, keeping in mind that CamKII interacts with L-type channels (Hudmon et al. 2005b; Grueter et al. 2008), these data suggest the existence of an ETC used to adapt $Ca_V1.2$ channels expression to intracellular calcium concentration via A calcium-calmodulin-CamKII sensor. This regulatory pathway also exists in neurons, since a calcium-insensitive dominant active form of DREAM induces a significant down-regulation of $Ca_V1.2$ channels in the cortex of transgenic mice (Naranjo and Mellström 2012). Finally, in cerebellar granule cells, it has been reported that DREAM interacts both with T-type calcium channels and K_V4 potassium channels(Anderson et al. 2010); these interactions allow calcium regulation of the K_V4 current. It would be worth testing to determine if specific electrical stimulation could unbind DREAM from T-type channels.

Presynaptic calcium channels have been shown to bind to several adaptor proteins. In particular, the PDZ protein MINT binds both to $Ca_V2.1$ and $Ca_V2.2$ channels, whilst CASK, a synaptic scaffolding protein, interacts exclusively with $Ca_V2.2$ channels. These interactions are implicated in the formation of the large macromolecular complexes which anchor the synaptic vesicle to the secretory machinery (Maximov et al. 1999). Moreover, it has been shown that MINT also interacts with CASK (Tabuchi et al. 2002; Zamponi 2003). Using imaging and biochemical approaches, CASK has been localized to the nucleus of neurons from embryonic brain. CASK regulates reelin gene expression by acting as a co-activator of the transcription factor Trb1 (Hsueh et al. 2000). It would be interesting to test if CASK is able to translocate from the synapse to the nucleus and if this process is triggered by calcium influx through Ca_V2 channels.

8.5 The Slow Protein Track: Unbinding of Ca_V Partners or Ca_V Fragments

A new aspect of VGCC signaling to the nucleus has been explored recently. VGCC subunits and truncated Ca_V channel subunits have been reported to be localized in the nucleus and to participate in transcription regulation. Here we will focus on the latest finding concerning $Ca_V\beta4$ subunits and Ca_V ICDs. However, it is noteworthy that $Ca_V\beta3$ subunits have been observed in the nucleus, where they bind to nuclear proteins and regulate transcription factors (Béguin et al. 2006; Zhang et al. 2010; Tadmouri et al. 2012).

8.5.1 Nuclear Ca_V Fragments

One of the findings from VGCC purification studies was that Ca_V1 and Ca_V2 channels can be cleaved at the C-terminus tail (Gerhardstein et al. 2000; Hell et al. 1993; De Jongh et al. 1991), releasing free C-terminal fragments that remain associated with the channel and maintain regulatory roles (Gao et al. 2001; Fuller et al. 2010). However, recently Gomez-Ospina et al. (2006) identified a 75 kDa C-terminal fragment of $Ca_V1.2$ called CCAT that translocates to the nucleus of inhibitory cortical neurons; CCAT interacts with nuclear proteins implicated in regulation of transcription-like thyroid hormone receptor, retinoic acid receptor and protein p54(nrd)/NonO. CCAT regulates the expression of numerous genes including the connexin *Cx31.1* gene (Gomez-Ospina et al. 2006). Moreover CCAT binds to the Cx31.1 promoter and is able to drive expression of a luciferase construction containing the promoter of *Cx31.1* gene. Finally, CCAT was shown to be a transcription factor in its own right, independently of $Ca_V1.2$ channels (Gomez-Ospina et al. 2006). How CCTA is generated is a remaining question, although a proteolytic cleavage of $Cav_V1.2$ seems to be the most likely process; however, the protease and the exact cleavage site needs to be found. Whatever the precise mechanism, it appears that the concentration of CCAT in the nucleus is regulated by calcium influx through L-type VGCCs and partially by others source of calcium such as NMDA receptors. Increasing electrical activity induces an export of CCAT from the nucleus (Gomez-Ospina et al. 2006). In cardiomyocytes, CCAT interacts with the *cacna1c* promoter and induces a repression of $Ca_V1.2$ expression, suggesting an autoregulatory mechanism of $Ca_V1.2$ channel expression (Schroder et al. 2009).

Using a specific antibody against the $Ca_V2.1$ C-terminal, Kordasiewicz et al. (2006) have clearly established that in neurons and in heterogeneous systems $Ca_V2.1$ is cleaved and produces a 60 kDa C-terminal fragment which is translocated to the nucleus. The $Ca_V2.1$ C-terminus has four successive putative NLS, of which only the first three seems to be required for nuclear localization (Kordasiewicz et al. 2006). Like CCAT, it has been suggested that the $Ca_V2.1$ C-terminal fragment is

implicated in gene regulation (Du et al. 2009), even if the mechanism of such regulation remain unclear. However, the $Ca_V\beta4$ is reported to be the $Ca_V\beta$ subunit with the highest affinity for $Ca_V2.1$ channels and to co-localize with them (De Waard et al. 1995; Bichet et al. 2000; Wittemann et al. 2000; Xie et al. 2007). In particular, the $Ca_V2.1$ C-terminal interacts with a specific $Ca_V\beta4$ region (Walker et al. 1998, 1999). The fact that $Ca_V\beta$ subunits are able to unbind from the Ca_V subunits (Bichet et al. 2000; Cantí et al. 2001; Restituito et al. 2001) and that $Ca_V\beta4$ subunits have a nuclear localization, leaves open the possibility that, after $Ca_V2.1$ cleavage, the $Ca_V2.1$ ICD remains associated with $Ca_V\beta4$. Considering the formation of any $Ca_V2.1$ ICD/$Ca_V\beta4$ dimer could facilitate the understanding of the mechanism of nuclear translocation and the role in the transcription of the $Ca_V2.1$ ICD.

8.5.2 Nuclear $Ca_V\beta$ Subunits

The $Ca_V\beta$ subunit is necessary for numerous functions of Ca_V. In particular, the $Ca_V\beta$ subunit is mandatory for proper targeting, regulation of activity and modulation of regulatory pathways which adapt the activity of Ca_V to the cell demand (Cens et al. 1998; Restituito et al. 2000; Rousset et al. 2003; Leyris et al. 2009; Buraei and Yang 2010). This explains why $Ca_V\beta$ subunits were first considered pure cytoplasmic and sub-plasma membrane proteins. However, later experiments showed that overexpressed GFP tagged $Ca_V\beta1$, $Ca_V\beta3$ and $Ca_V\beta4$ subunits are localized in the nucleus of adult cardiomyocytes (Colecraft et al. 2002). Subsequently, overexpressed and endogenous $Ca_V\beta$ subunits have been found in the nucleus of multiple excitable cells. In particular, nuclear localization of endogenous $Ca_V\beta4$ has been observed in NG108 cells, Purkinje neurons, cerebellar granular cells, dorsal cochlear nucleus neurons, medial vestibular nuclei neurons, hippocampal neurons and myotubes (Subramanyam et al. 2009; Xu et al. 2011; Tadmouri et al. 2012). A systematic comparison of nuclear targeting of $Ca_V\beta$ subunits showed that the $Ca_V\beta4b$ subunit has the highest nucleocytoplasmic ratio (Subramanyam et al. 2009). Analysis of $Ca_V\beta$ subunits sequences showed that they possess a nuclear export sequence (NES), but are devoid of a NLS. These analyses failed to disclose any advantageous structural element, which could explain the strong nuclear tropism of $Ca_V\beta4b$. However, truncation of the $Ca_V\beta4b$ N-terminal reduced nuclear targeting, pointing the importance of this region; conversely, addition of the $Ca_V\beta4b$ N-terminal segment (amino acids 1–48) to $Ca_V\beta2a$ increased its nucleocytoplasmic ratio to values similar to those of $Ca_V\beta4b$ (Subramanyam et al. 2009). A stretch of basic residues (RRSRLKR) located between the position 28 and 34 of $Ca_V\beta4b$ played a key role as the mutation of amino acids R28-R29-S20 in A28-A29-A30 induced a drastic reduction of the nucleocytoplasmic ratio, close to the level observed with $Ca_V\beta4a$ and $Ca_V\beta3$ (Subramanyam et al. 2009); therefore, this sequence contributes to the large nuclear translocation specifically observed with the $Ca_V\beta4b$ subunit.

Nevertheless, other sequences are equally important to elicit nuclear targeting of $Ca_V\beta4b$ subunits. The truncation of the last 38 amino acids decreased drastically the $Ca_V\beta4b$ subunit nucleocytoplasmic ratio (Tadmouri et al. 2012). This sequence is in fact one of the binding sites for the protein B56δ, a binding partner of $Ca_V\beta4b$ and $Ca_V\beta3$ identified in a two-hybrid screen (Tadmouri et al. 2012). B56δ is a regulatory subunit of the protein phosphatase 2a (PP2a), which is a heterotrimeric serine/threonine phosphatase. B56δ has a NLS in its C-terminal part, which is sufficient to translocate endogenous $Ca_V\beta4b$ to the nucleus of undifferentiated NG108 cells. Moreover in a context of B56δ down regulation, the nuclear localization of $Ca_V\beta4b$ is impaired. It is interesting to note that the integrity of the two conserved domains SH3 and MAGUK of $Ca_V\beta4b$ is required to allow its interaction with B56δ. These two domains bind to each other and the alteration of this interaction prevented B56δ binding to $Ca_V\beta4b$ (Tadmouri et al. 2012). Thus, it appears that multiple structural determinants are essential for nuclear translocation of $Ca_V\beta4b$. This suggests the possibility that these sequences do not work together, but are more probably recruited individually according to conditions and cell types. $Ca_V\beta4b$ subunits may use different pathways to enter the nucleus, some being specific and some being shared with others $Ca_V\beta$ subunits.

What is the role of $Ca_V\beta4$ subunits in the nucleus? A first clue was provided by studies on a short isoform of the $Ca_V\beta4$ subunit, the $Ca_V\beta4c$ subunit, expressed in cochlear hair cells, brainstem neurons and heart (Hibino et al. 2003; Xu et al. 2011). $Ca_V\beta4c$ is produced by skipping exon 9 of *cacnb4* gene which creates a frameshift and a premature stop codon. The corresponding protein is truncated at the beginning of the conserved guanylate-kinase (GK) domain and exhibits an additional specific sequence of 13 amino acids at the C-terminus. Using two-hybrid approaches, $Ca_V\beta4c$ have been shown to interact with the three members of the HP1 family (Hibino et al. 2003). HP1 are heterochromatin binding proteins structured in three parts: a chromodomain (CD) and a chromo shadow domain (CSD) separated by a linker that interacts with DNA. While CSD is a protein-protein interaction domain which binds to various nuclear proteins including SUV39H1/2, CD binds the methylated Lysine in position 9 on the histone H3 C-terminal. The lysine H3K9 is tri-methylated by diverse methytransferases including SETDB1 and SUV39H1/2; binding of HP1 to H3K9me is a critical step in the formation and maintenance of heterochromatin structure (Zeng et al. 2010). Heterochromatin is a specific organization of the DNA-histone complex which is inaccessible to the transcriptional machinery; accordingly, this chromatin state represses transcription of the large chromosomal domain. HP1 proteins, which are also able to silence individual genes by H3K9me binding, are markers of epigenetic silencing. Interestingly, all four $Ca_V\beta$ subunits possess a binding site for HP1, namely the short sequence PVVLV; for example, located at position 187–191 of $Ca_V\beta4b$, which is very similar to the HP1 binding consensus motif, PxVxL, found on others HP1 partners, such as the chromatin assembly factor 1. However $Ca_V\beta4c$ is the only $Ca_V\beta$ subunit shown to interact with HP1γ. This differential affinity of $Ca_V\beta$ subunits has been confirmed using the GAL4-CAT reporter assay, and it has been shown that $Ca_V\beta4c$, but not

$Ca_V\beta4a$, diminished the HP1γ silencing effect on a GAL4-CAT artificial gene (Hibino et al. 2003). It has been proposed that PVVLV sequence in full length $Ca_V\beta$ subunit is buried in a β-strand of the GK domain and is not accessible to HP1 interaction, explaining why the $Ca_V\beta4a$ subunit is not able to interact with HP1 (Xu et al. 2011). An important point is that binding between HP1 and $Ca_V\beta4c$ is mandatory for the nuclear targeting of $Ca_V\beta4c$.

Is the full-length $Ca_V\beta4b$ also implicated in gene silencing? Imaging experiments using electronic microscopy indicate that ∼50 % of the nuclear $Ca_V\beta4b$ is associated with heterochromatin, suggesting a potential role in the regulation of chromatin state (Tadmouri et al. 2012). Tadmouri et al. have shown that the interaction between HP1γ and the full-length beta subunit is more subtle than thought previously. The $Ca_V\beta4b$ is in fact able to bind to HP1γ, but only if B56δ is already attached to the $Ca_V\beta4b$ subunit. This indicates that the binding of B56δ opens the secondary structure of the $Ca_V\beta4b$ MAGUK domain, which correlates with the requirement of an intact SH3/MAGUK interaction for the B56δ binding to $Ca_V\beta4b$.

As HP1 proteins bind to the nucleosome, it would be interesting to determine if the $Ca_V\beta4b$/B56δ/HP1γ complex also binds to the nucleosome. Immunoprecipitation experiments have revealed that overexpressed and endogenous $Ca_V\beta4b$ are able to interact with histones H2, H3 and H4. This interaction is mediated by B56δ as the strength of the histone/$Ca_V\beta4b$ interaction is correlated with the level of B56δ expression (Tadmouri et al. 2012). Additionally $Ca_V\beta4b$ lacks capacity to interact with the histone H3 in B56δ knockout mice. It is known that the binding of HP1γ to chromatin during the cell cycle requires a tri-methylation of H3K9 and is regulated by the phosphorylation state of H3S10 (Fischle et al. 2005; Hirota et al. 2005; Terada 2006). AuroraB which phosphorylates H3S10 at the start of mitosis, lowers the affinity of HP1γ for chromatin; however, during interphase H3S10phos is dephosphorylated, which induces re-association of HP1γ with chromatin, a mechanism termed a "binary methylation-phosphorylation switch" (Dormann et al. 2006). B56δ is a regulatory subunit of the PP2A phosphatase and PP2A may dephosphorylate H2S10phos (Nowak et al. 2003; Simboeck et al. 2010). Moreover, $Ca_V\beta4b$ is able to immunoprecipitate PP2A in the presence of B56δ and immunoprecipitation of the $Ca_V\beta4b$/B56δ/PP2A/HP1γ complex induces dephosphorylation of an 8 amino acid histone3 peptide that contains a phosphorylated serine 10 site (Tadmouri et al. 2012). Together, these data suggest that $Ca_V\beta4b$/B56δ/PP2A/HP1γ is a functional complex in which PP2A allows HP1γ binding to the nucleosome, whilst HP1γ mediates heterochromatization.

Is the $Ca_V\beta4b$/B56δ/PP2A/HP1γ complex acting in a broad, non-specific way or at a specific site on DNA? The *lethargic* mouse, considered as a spontaneous knock out of the $Ca_V\beta4$ subunit, displays neurological disorders including ataxia and epilepsy (Burgess et al. 1997). The analysis of *lethargic* mice cerebellum and forebrain transcriptomic profiles revealed the expression of more than 50 genes showing a significantly change, the *Th* gene being the most increased (Tadmouri et al. 2012). Since 80 % of genes are up-regulated, $Ca_V\beta4$ seems to have an overall silencing impact. Their large distribution over all of the genome suggests that the $Ca_V\beta4b$/B56δ/PP2A/HP1γ complex may inhibit individual genes, rather

than acting by a regional silencing effect. Since the $Ca_V\beta$ subunit has no DNA binding sequence, we speculate that $Ca_V\beta4$ must bind others proteins, such as transcription factors able to target specific genes. During a two-hybrid screen, an interaction of $Ca_V\beta4b$ with the transcription factor thyroid hormone receptor alpha (TRα) has been found (Tadmouri et al. 2012). TRα usually binds to DNA via a TRE consensus site in the absence of the hormone and represses gene expression; binding of T3 hormone to TRα induce a conformational change of the receptor which become able to recruit the transcription machinery and to initiate gene expression (Cheng et al. 2010). TRα can bind constitutively to the *Th* promoter, even in absence of $Ca_V\beta4$ or B56δ. Contrary to the canonical view, T3 hormone application induced a repression of luciferase expression under the control of the *Th* promoter. Interestingly co-expression of the $Ca_V\beta4$ subunit turns TRα receptor into a mere repressor, independently of the presence of T3 hormone (Tadmouri et al. 2012). Chromatin immunoprecipitation experiments demonstrate that beside TRα, $Ca_V\beta4b$, HP1γ, B56δ and PP2A interact with the *Th* promoter (Tadmouri et al. 2012). Using the "Promoter Analysis and Interaction Network Generation Tool" called PAINT (Vadigepalli et al. 2003; Gonye et al. 2007), we retrieved the 5' sequence from the entire *Lh* mice modulated gene set and listed all transcriptional regulatory elements present on these cis-regulatory regions. We found that several predicted transcription factor binding sites are over-represented. C-rel and Pax-6 were the most significantly over-represented transcription factor. Pax-6 has been already reported to bind to $Ca_V\beta$ subunits (Zhang et al. 2010) and we confirmed the interaction of $Ca_V\beta4$ with c-rel using imaging and biochemical approaches (Bellis et al. in preparation). Our theory is that binding of $Ca_V\beta4$ to transcription factors brings the silencing machinery B56δ/PP2A/HP1γ to specific site(s) on DNA and represses expression of the corresponding genes.

Is nuclear $Ca_V\beta$ subunit due to $Ca_V\beta$ acting alone or are there messengers between VGCC and nucleus? In cerebellar granular neurons, *Th* expression is repress by activity; this effect is mediated by the $Ca_V\beta4$ subunit since *Th* gene expression become activity-independent in *Lh* mice (Tadmouri et al. 2012). Moreover, the nuclear localization of $Ca_V\beta4b$ is regulated by calcium influx and the VGCC (Subramanyam et al. 2009; Tadmouri et al. 2012). Biochemical studies have shown that B56δ and PP2A binding to $Ca_V\beta4b$ is sensitive to activity in cultured neurons and occurs only after strong chronic depolarization in heterogeneous systems which over-express $Ca_V\alpha$ subunits (Tadmouri et al. 2012). These results suggest a binding competition to $Ca_V\beta$ subunits between $Ca_V\alpha$ and B56δ that is regulated by the excitability state of the neuron, bringing new credence to the possible unbinding of $Ca_V\beta$ from $Ca_V\alpha$ subunits (Restituito et al. 2001). We speculate therefore that $Ca_V\beta4$ is a messenger which, during certain excitability episodes, unbinds from the $Ca_V\alpha$ subunit, translocates to the nucleus with B56δ and targets specific transcription factors associated with gene promoters.

The physiological significance of $Ca_V\beta4$ nuclear localization is still under investigation. In some case, the nuclear localization of $Ca_V\beta4b$ is developmentally regulated. For example, in NG108 cells, the $Ca_V\beta4$ nucleocytoplasmic ratio increases gradually as differentiation takes place. Heterochromatin plays a pivotal

role during the development and differentiation of cells. Recent data obtained in zebrafish point out such roles of $Ca_V\beta4$ in development, with the appearance of a lethal phenotype when $Ca_V\beta4$ is down regulated (Ebert et al. 2008).

8.6 Concluding Remarks

Chronic depolarization of hippocampal neurons using >60 mM KCl induces synchronously nuclear translocation of NFAT4c, MAPK/CREB pathway activation, CAMK/CREB activation and the nuclear translocation of the $Ca_V\beta4b$ subunit released from the VGCC. This example illustrates the role of VGCC as a nuclear signaling platform able to trigger a large diversity of signals to the nucleus. However, such KCl stimulation protocols mask a probable finer correlation between excitability events and the signals triggered by VGCC. Each depolarization amplitude or stimulation frequency generates specific signaling pathway, as suggested by the differential activation thresholds of MAPK/CREB and CamK/CREB pathways. Another element participating in the decoding of signals issued from the VGCC is the proteome of the channel. VGCC are not only a calcium source, but also a signaling hub where numerous calcium sensors and signaling proteins are part of the macromolecular complex organized around the channel. However, it is likely that VGCCs can be divided into several sub-groups, dependent on function, subcellular localization and their proteome. In consequence, proteomes of $Ca_V1.2$ channels localized at the dendritic shaft or in the soma should have a large number of common partners, but also few specific key partners. As already suggested, a calcium channel inserted into specific slot receives specific electrical stimulations and is surrounded by a specific set of proteins, combinations of which define a specific type of biological response (Cao et al. 2004). Subsequently, in theory, if NFAT4c nuclear translocation, MAPK/CREB activation, CAMK/CREB activation and/or nuclear translocation of $Ca_V\beta4b$ subunits could occur synchronously from a single $Ca_V1.2$ channel, it would be more realistic that each $Ca_V1.2$ channel slot is specialized in coupling excitation to a restricted number of signaling cascades.

At the same time, it is clear that several types of VGCCs are able to initiate the same pathway, or pathways, which converge on a common transcriptional effector, such as CREB. Again, by integrating the biophysical specificity of each channel type, the neuron is able to respond to a range of potential signals by recruiting signaling cascades common to most gene remodeling events. The key differences come from the coupling efficiency of the signaling molecules. Both Ca_V1 and Ca_V2 calcium channels are able to initiate the CamK/CREB pathway, but different spatial modes of activation differentiate them. Whilst Ca_V1 channels group all the proteins required to activate the CamK/CREB signaling cascade in their nanodomain, Ca_V2 channels act in less specific way at the micrometer scale via local calcium increases. Accordingly, Ca_V2 channels require a larger, more sustained stimulation to induce a sufficient local calcium rise required to activate local CamK. In consequence, the activated CamK pool will be dependent on the strength of the stimulation which,

in turn, will be interpreted differentially at the nuclear level and lead to different biological outputs.

The neuronal nucleus receives multiple waves of signals which add a temporal dimension to VGCC-nucleus communication. From an initial stimulation episode, several messengers are translocated to the nucleus with different kinetics and thus arrive at the nucleus at different times. The best known example is the L-type VGCC which lead to CREB phosphorylation at serine 133 via two independent signaling pathways with different time frames. The first pathway, which uses CamK activation and is carried by calcium waves, is very quick and lasts less than 15 min, whilst the second pathway, which uses MAPK is slower and requires nuclear translocation of signaling proteins. The distinct properties between these two pathways are certainly of primary importance for the computational properties of neurons. For example, these superposed signals can provide information about the strength of the electrical stimulation. For small amplitude depolarizations, only CamK/CREB can be activated, whilst larger depolarization involve both signaling pathways which, in turn, changes the life time of phosphorylated CREB and results in different activity-dependent transcription programs (Liu and Graybiel 1996).

This temporal distinction can also provide information about the spatial scope of the stimulation. It is assumed that calcium wave propagation is a mechanism that causes an amplification of the signal, unlike the physical nuclear translocation system which de facto reveals the number of VGCC recruited by the stimulation. Accordingly, fast track signaling gives information regarding the local stimulation input that requires a rapid but non-specific change of gene expression, whilst slow track signaling works in a cooperative fashion to integrate multiple channel recruitment and converges on the nucleus, where the signal leads to a more profound gene remodeling. $Ca_V\beta$ subunits translocate to the nucleus by inducing change in chromatin status, which is believe to represent longer term changes to gene expression.

It is also known that CREB can be phosphorylated at others sites. In particular, serine 142 and serine 143 are phosphorylated specifically after calcium influx, an effect which occurs with a delay after serine 133 phosphorylation (Gau et al. 2002; Kornhauser et al. 2002). This delayed phosphorylation inhibits the binding of the CBP protein and down regulates CRE-dependent transcription. Phosphorylation of serine 142 is sensitive to KN-93, but not to PD-98059, which means that CamKs are specifically implicated (Gau et al. 2002; Kornhauser et al. 2002). We can speculate that different kinase pathways triggered by L-type channels activation may target different phosphorylation sites on CREB, additional to serine 133, and thus regulate CRE-dependent transcription. Finally, Dolmetsch et al. have demonstrated that MAPK pathway activation by calcium influx through L-type channels was also able to activate MEF, another activity-dependent transcription factor (Dolmetsch et al. 2001). Besides CREB, the two delayed kinase pathways activated by L-type channels can also target different sets of activity-regulated transcription factors that activate, in turn, specific and non-overlapping transcription programs (Benito et al. 2011).

8.7 Physiopathological Perspectives

If the different pathways implicated in activity-dependent gene regulations are now relatively well-defined, the gene programs initiated and their effects on neurons are still in debate. An entry point is to consider the physiopathological aspects of VGCC-nucleus communication. It has been mentioned elsewhere that many inherited neurological disorders present mutations in proteins engaged in activity-dependent-transcription pathways (Deisseroth and Tsien 2002; Greer and Greenberg 2008; Ulrich et al. 2012). Rubenstein-Taybi syndrome is a mental retardation disease caused by mutation in the CBP gene (Petrij et al. 1995). Coffin-Lowry syndrome is due to a mutation in the gene coding for rsk2, one of multiple kinases participating to the phosphorylation of CREB at the serine 133 (Trivier et al. 1996). The calcineurin/NFATc4 pathway has been implicated in β-amyloid-neurotoxicity (Wu et al. 2010; Hudry et al. 2012). Some polymorphisms in the *bdnf* gene, which is an IEG gene, results in memory deficit (Chen et al. 2006). Similarly, it has been shown that mutations in the gene *cacnb4* coding for $Ca_V\beta4$ are responsible of epilepsy and ataxia. At least two mutations have been identified, the mutation L125P in the middle of the SH3 domain and the R481X at the C-terminus of the protein (Escayg et al. 2000). Electrophysiological characterization did not give any clues in the pathogenesis of the disease; however, Tadmouri et al. have demonstrated that $Ca_V\beta4b$ harboring the identified mutation lost their ability to bind to B56δ and to be translocated to the nucleus (Tadmouri et al. 2012). Undeniably, this lead has to be investigated more deeply to determine the potential link between the chromatin remodeling mediated by $Ca_V\beta4b$ and the neuronal change required to avoid epileptic events. One initial approach would be to determine all the binding sites on DNA for the $Ca_V4\beta$/B56δ/PP2A/HP1γ complex, this would open the door to a systematic determination of the genes that are under the control of this complex. Moreover, the identification of the physiological conditions that drives the unbinding of $Ca_V\beta4b$ from the VGCC will provide pivotal knowledge to understand the pathogenesis of these diseases.

The $Ca_V2.1$ C-terminal fragment observed in the neuronal nucleus also constitutes a serious candidate for elucidation of spinocerebellar ataxia type 6 (SCA6) pathogenesis. This debilitating disease, characterized by a late onset and progressive Purkinje neurons loss, is due to an abnormal polyglutamine (poly-Q) expansion in the C-terminus of a $Ca_V2.1$ isoform. Interestingly, this poly-Q sequence is present in the $Ca_V2.1$ cleaved fragment identified in the nucleus. Recent results indicate that the $Ca_V2.1$ fragment is able to bind to the promoter sequence of at least three genes: BTG1, progranulin and PMCA2 (Du et al. 2009). This $Ca_V2.1$ fragment has been also shown to drive BTG1 expression; however, a $Ca_V2.1$ fragment version containing a pathological poly-Q sequence (33 successive glutamines) is unable to regulate BTG1 expression (Du et al. 2009). Despite the current absence of evidence supporting a link between ataxia pathogenesis and transcriptional regulatory role of the $Ca_V2.1$ fragment, it is clear that investigation of $Ca_V2.1$ fragment activity in the nucleus is warranted.

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Part III
Mechanisms of Studying Calcium Channel Effects

Chapter 9
Presynaptic Ca^{2+} Influx and Its Modulation at Auditory Calyceal Terminals

Holger Taschenberger, Kun-Han Lin, and Shuwen Chang

Abstract Calyx and endbulb synapses of the mammalian auditory brainstem are specialized in transmitting spike activity fast, sustained and temporally precise. To accomplish this task, they make use of unusually large presynaptic elements which form axosomatic contacts with their postsynaptic target neurons. The large size of the calyceal terminals represents a major experimental advantage and has enabled electrophysiologists to study the functional properties of Ca^{2+} channels in presynaptic CNS terminals in great detail, with high time resolution and unprecedented precision. Calyx and endbulb terminals express several thousands of Ca^{2+} channels with rapid kinetics which ensure fast and efficient gating of Ca^{2+} influx during brief action potentials. When repetitively activated, presynaptic Ca^{2+} influx is modulated in a frequency-dependent manner and this Ca^{2+} current modulation contributes significantly to short-term plasticity at these synapses. During short high-frequency bursts, Ca^{2+} influx is facilitated whereas tetanic activity or low-frequency firing leads to an accumulation of Ca^{2+} channel inactivation. When the calyx synapses mature, the coupling between docked transmitter vesicles and Ca^{2+} channels becomes tighter to compensate for the shortening of the presynaptic action potential duration. Many of the G protein-dependent pathways of Ca^{2+} channel regulation that are potent in immature synapses are weakened during postnatal development.

Keywords Endbulb of Held • Calyx of Held • Presynaptic function • Presynaptic Ca^{2+} channels • Short-term plasticity

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9.1 Introduction

Owing to their small size, presynaptic endings of the mammalian brain are generally inaccessible for direct electrophysiological recordings. Thus, our knowledge about the functional properties of presynaptic voltage-gated Ca^{2+} channels (VGCCs) and their regulation is still limited and in many cases has been derived indirectly for example by analyzing postsynaptic responses. Fortunately, there are a few exceptions to this rule. For example, within the mammalian auditory brainstem two glutamatergic axosomatic synapses with exceptionally large presynaptic terminals are found, which have long been known to anatomists for their distinctive and fascinating morphology (Held 1893; Ramón y Cajal 1911; Morest 1968). These are the calyx of Held synapse and its smaller cousin the endbulb of Held synapse. Both synapses participate in sound localization (Masterton et al. 1967) and their structural features appear to facilitate the temporally precise transmission of spike activity along the auditory pathway which is a prerequisite for various tasks of auditory information processing (Rhode and Greenberg 1992; Oertel 1999; Young and Oertel 2004). About 20 years ago, after demonstrating that direct recordings with patch pipettes are feasible at its terminal, calyx of Held synapses—and more recently endbulb synapses too—have been 're-discovered' by electrophysiologists as valuable model for studying presynaptic function (Forsythe 1994; Borst et al. 1995; Lin et al. 2011).

Endbulb and calyx synapses represent the second and third synapses along the binaural auditory pathway. This pathway is involved in computing sound source localization in the auditory space by detecting interaural timing and level differences of sound and synapses participating in this task are specialized for transmitting electrical signals fast and reliably. Endbulb synapses are formed between the endings of auditory nerve fibers and bushy cells (BCs) in the anterior ventral cochlear nucleus (AVCN). The axons of spiral ganglion cells convey auditory sensory information from the inner hair cells to spherical BCs (SBCs) via large calyx-type axosomatic terminals—the endbulbs of Held. SBCs carry timing information to the medial superior olivary nuclei, where the arrival time of sounds at the two ears is compared. Detailed reconstructions of endbulb → SBC connections have revealed that up to four endbulb terminals can contact single bushy cells (Fig. 9.1b) (Brawer and Morest 1975; Ryugo and Sento 1991; Nicol and Walmsley 2002). Each endbulb terminal can harbor many active zones with large clusters of synaptic vesicles (Neises et al. 1982; Ryugo et al. 1996, 1997; Nicol and Walmsley 2002).

The calyx of Held synapse is formed by fibers originating from the globular bushy cells (GBCs) of the AVCN and terminating onto principal cells of the medial nucleus of the trapezoid body (MNTB) which is situated ventromedial to the medial superior olive (MSO). Each principal neuron is contacted by only a single presynaptic fiber that ends in the form of a cup-like termination—the calyx of Held—which embraces approximately two thirds of the principal cell's membrane surface (Fig. 9.1a) (Morest 1968). Among glutamatergic synapses of the mammalian brain, the calyx of Held has without a doubt become one of the

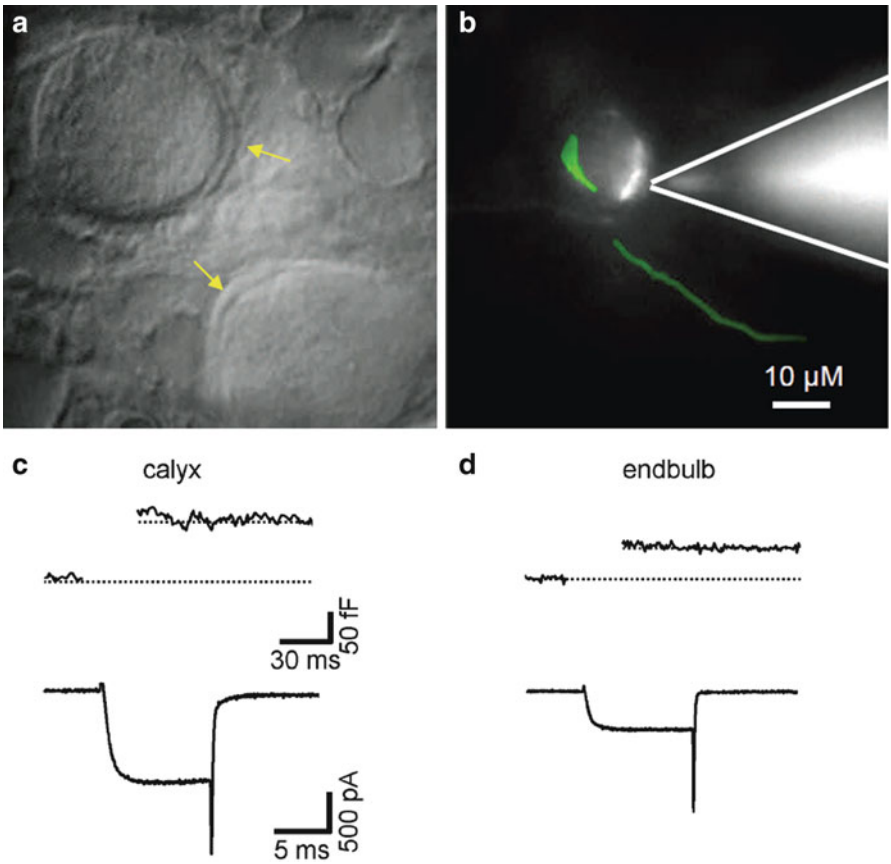

Fig. 9.1 Identification of calyx and endbulb terminals. Two hundred μm thick slices were prepared from brainstems of a postnatal day 9 rat (**a**) or mouse (**b**). (**a**) MNTB principal neurons visualized using differential interference contrast. Each principal cell is surrounded by a single giant presynaptic terminal—the calyx of Held (*arrows*). (**b**) Bushy cells of the anterior ventral cochlear nucleus (AVCN) are contact by several large endbulb terminals. Here, two terminals contacting a single bushy cell were sequentially loaded with a fluorescent dye via a presynaptic patch pipette (*white lines*). (**c**, **d**) Changes in membrane capacitance (ΔC_m, *top*) elicited by step-depolarizations (10 ms, from $V_h = -80$ to 0 mV) recorded from a calyx (**b**) and an endbulb (**d**) terminal. The corresponding $I_{Ca(V)}$ are shown in the *bottom panel*

best characterized (for review see Schneggenburger et al. 2002; von Gersdorff and Borst 2002; Meinrenken et al. 2003; Schneggenburger and Forsythe 2006; Borst and Soria van Hoeve 2012). This derives primarily from the unusual large size of its presynaptic terminal which, therefore, renders it accessible to patch pipettes (Forsythe 1994; Borst et al. 1995; Takahashi et al. 1996; Chuhma and Ohmori 1998; Sun and Wu 2001). Because of its giant size, the calyx terminal harbors several hundreds of release sites, allowing it to drive the principal neurons reliably at

high frequencies. The principal neurons of the MNTB are glycinergic and provide precisely timed and sustained inhibition to many other auditory brainstem nuclei. The calyx of Held synapse thus functions as a fast, reliable, inverting relay synapse.

9.2 General Characteristics, Number and Distribution of Presynaptic VGCCs

During patch-clamp experiments in brainstem slices, endbulb and calyx terminals are easily identifiable (Fig. 9.1a, b) and the presynaptic origin of the recordings can conveniently and unequivocally be verified by monitoring transmitter release after depolarization-induced Ca^{2+} influx. The fusion of transmitter vesicles leads to an transient increase in membrane area, which can be measured as an increase in surface membrane capacitance (Jaffe et al. 1978; Gillespie 1979; Neher and Marty 1982; Lin et al. 2011) (Fig. 9.1c, d). When pharmacologically isolated, inward whole-cell Ca^{2+} currents ($I_{Ca(V)}$) with peak amplitudes typically between 0.5 and 2.5 nA can be recorded in mouse or rat calyx of Held terminals with 2 mM external Ca^{2+} (Borst et al. 1995; Borst and Sakmann 1996, 1998b; Chuhma and Ohmori 1998; Forsythe et al. 1998). The presynaptic $I_{Ca(V)}$ appears to be relative resistant to run-down after internal dialysis has begun, at least in the presence of internal ATP and ATP-regenerating substrates such as phosphocreatine. The average $I_{Ca(V)}$ in endbulb terminals is about two thirds smaller than that of the calyx which is consistent with the much smaller size of the endbulb terminals (Lin et al. 2011) (Fig. 9.1 c, d). Despite the considerable variability of $I_{Ca(V)}$ amplitudes among individual calyx and endbulb terminals, a relatively robust linear correlation between $I_{Ca(V)}$ and the membrane capacitance is observed indicating a rather similar average current density of ~0.076 nA/pF and ~0.1 nA/pF in calyx and endbulb terminals, respectively (Fig. 9.2) (Lin et al. 2011). The presynaptic $I_{Ca(V)}$ shows the typical bell-shaped I-V relationship: it activates at membrane potentials more positive than −40 mV and peaks between −10 and 0 mV. The presynaptic Ca^{2+} channels show remarkably little steady state inactivation (Fig. 9.3) (Forsythe et al. 1998; Lin et al. 2011) and, in contrast to the bushy cell bodies, neither calyx nor endbulb terminals express low-voltage activated, transient (T-type) Ca^{2+} currents. One may argue that failsafe transmission at these synapses requires relative insensitivity to fluctuations of the presynaptic resting membrane potential, and T-type Ca^{2+} channels would be especially ill-suited for the purpose of triggering action potential-evoked presynaptic Ca^{2+} influx reliably.

Noise analysis of $I_{Ca(V)}$ amplitude fluctuations provided estimates for the apparent single channel current of ~0.08 pA at a membrane potential of 0 mV, suggesting that on average >6,000 and >16,000 open channels contribute to generating average current amplitudes of 0.5 and 1.3 nA in endbulb and calyx terminals, respectively (Lin et al. 2011). These single channel current estimates are slightly smaller than those obtained at hippocampal mossy fiber boutons (0.13 pA

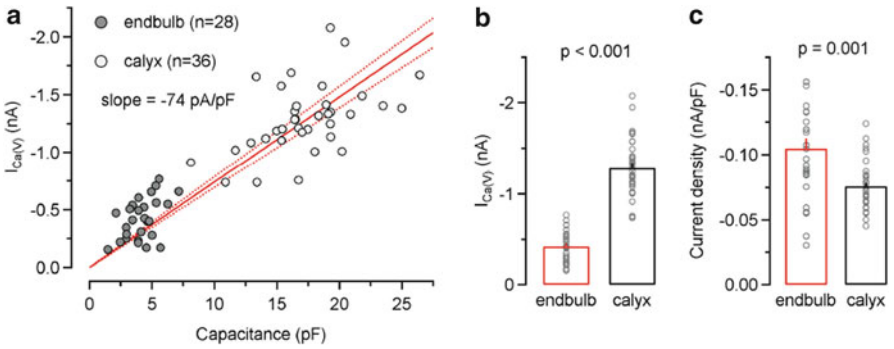

Fig. 9.2 Ca^{2+} current densities are slightly larger in endbulb versus calyx terminals. (**a**) Scatter plot of $I_{Ca(V)}$ versus terminal capacitance obtained from 28 endbulb and 36 calyx terminals. *Solid and dotted red lines* represent linear regression and 95 % confidence intervals for the entire data set, respectively. The slope of the regression line is −74 pA/pF. (**b**, **c**) Average amplitudes (**b**) and current densities (**c**) of $I_{Ca(V)}$ in endbulb and calyx terminals (Modified from Lin et al. 2011)

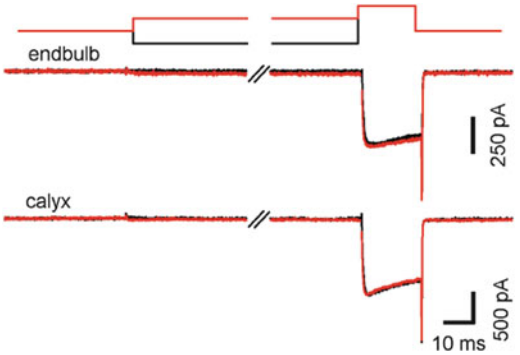

Fig. 9.3 Negligible steady-state inactivation of $I_{Ca(V)}$. $I_{Ca(V)}$ was elicited in endbulb (*middle traces*) and calyx (*bottom traces*) terminals by 20 ms pulses to 0 mV after conditioning depolarization (500 ms duration) to −120 mV (black) or −40 mV (*top traces*) (Modified from Lin et al. 2011)

at 0 mV) (Li et al. 2007). Using slightly elevated external Ca^{2+} (10 mM), larger single channel currents (0.27 pA) were measured recently by directly recording in the cell-attached configuration from the terminal's release face that was exposed by applying positive pressure in brainstem slices pretreated with the protease papain (Sheng et al. 2012). Nevertheless, it is conceivable that single endbulb and calyx terminals harbor several thousands of VGCCs.

How are these channels distributed over the presynaptic membrane? Whilst ultrastructural data is still missing, some insights about the spatial organization of Ca^{2+} channels can be gained from dual patch-clamp recordings at the calyx of Held. When monitoring transmitter release after direct presynaptic depolarizations with voltage commands that cause the opening of a variable number of Ca^{2+} channels, the EPSC size changes in a supralinear manner. It was therefore concluded that the majority of readily releasable vesicles at the calyx is controlled by several Ca^{2+} channels per vesicle (Borst and Sakmann 1999). This contrasts the situation at

the squid giant synapse (Augustine et al. 1991), the frog neuromuscular junction (Yoshikami et al. 1989) and the chick ciliary ganglion synapse (Stanley 1993), where the release of synaptic vesicles seems to be controlled by only a single Ca^{2+} channel. Assuming that the vast majority of Ca^{2+} channels are concentrated at active zones and considering the total number of readily releasable vesicles in endbulb and calyx synapses, one arrives at an estimate of approximately 6–8 VGCCs that are associated with each docked vesicle (Lin et al. 2011). Cell-attached patch recordings from the release face of calyx terminals revealed that single active zones contain an average number of ~40 VGCCs (Sheng et al. 2012). No Ca^{2+} currents are detected in patches from the calyx membrane not opposed to the postsynaptic neuron reinforcing the notion that VGCCs must be clustered at the release face (Sheng et al. 2012).

Immature calyces of Held ($\leq$ postnatal day (P) 12) express a mixture of N-, R-, and P/Q-type Ca^{2+} channels among which P/Q-type channels couple more efficiently to release than the other types do, suggesting that they are more concentrated at active zones (Wu et al. 1999). In contrast, endbulb terminals nearly exclusively express Ca^{2+} channels of the P/Q-type (Lin et al. 2011). The same applies for calyces of post-hearing mice and rats suggesting a developmental shift in the expression of their α-subunits (see below) (Iwasaki and Takahashi 1998; Iwasaki et al. 2000).

9.3 Ca^{2+} Influx During Presynaptic Action Potentials

Borst and Sakmann (1998b) measured the presynaptic $I_{Ca(V)}$ during an action potential (AP) waveform using two-electrode voltage-clamp at juvenile (P 8–10) rat calyces. These experiments revealed important information about magnitude and timing of the presynaptic Ca^{2+} influx in a mammalian CNS terminal. The AP-evoked $I_{Ca(V)}$ starts shortly after the peak of the calyceal AP and ends before the terminal is fully repolarized. This leads to a rapid rise in the global intracellular free Ca^{2+} ($[Ca^{2+}]_i$) with a rise time of ~0.32 ms (Habets and Borst 2006). The rapid gating kinetics of Ca^{2+} channels in the calyx of Held allows the AP to open the channels quite effectively such that the peak $I_{Ca(V)}$ during the AP repolarization phase is ~70 % of its maximum possible size. The AP-driven $I_{Ca(V)}$ can be well described by a Gaussian function having a peak amplitude of about −2.5 nA and a half-width of ~0.36 ms. The current integral is about −0.92 pC (Borst and Sakmann 1998b). At near physiological temperature, peak amplitudes almost double compared to recordings obtained at room temperature. However, because the AP half-width decreases by approximately 50 % when raising temperature, the total Ca^{2+} influx is actually smaller at near physiological compared with room temperature.

Endbulb APs are slightly smaller in amplitude and shorter in duration compared to calyx APs and thus open presynaptic Ca^{2+} channels less effectively. Because of their much smaller size, two-electrode voltage-clamp is not feasible at endbulb

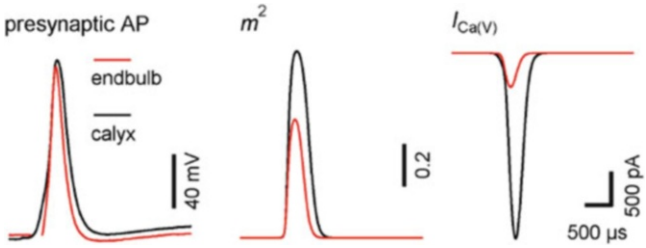

Fig. 9.4 Comparison of simulated $I_{Ca(V)}$ during presynaptic endbulb and calyx APs. *Left*, Endbulb APs (*black*) are slightly smaller in amplitude and shorter in duration compared to those recorded from calyxes (*gray*). V_m was −80 mV. *Middle*, Simulated time course of the activation parameter m^2. Because of its larger amplitude and longer duration, the calyceal AP waveform opens presynaptic VGCCs more efficiently than the endbulb AP. *Right*, Simulated $I_{Ca(V)}$ during presynaptic APs in endbulb (*black*) and calyx (*gray*). On average, the simulated presynaptic $I_{Ca(V)}$ is ∼6 times smaller for endbulb compared to calyx terminals (Modified from Lin et al. 2011)

terminals. Using the Hodgkin-Huxley formalism to model AP-driven Ca^{2+} influx, it was shown that the open probability (m^2 activation parameter) peaks at a considerably lower value in endbulb (0.49) compared to calyx (0.88) terminals of mice of the same age, even though the Ca^{2+} channel gating kinetics is of similar speed in both types of terminals (Lin et al. 2011). Together with the much lower number of Ca^{2+} channels expressed in endbulb terminals this results in a simulated $I_{Ca(V)}$ of comparably rapid kinetics but with ∼6 times smaller amplitude (−0.45 vs. −2.8 nA; Fig. 9.4) (Lin et al. 2011).

9.4 Inactivation of Presynaptic VGCCs

During prolonged presynaptic depolarizations, $I_{Ca(V)}$ inactivates with a double-exponential time course (Forsythe et al. 1998; Lin et al. 2012). The fast component of inactivation has a time constant of ∼35 ms and appears to be Ca^{2+}-dependent as it is greatly reduced when Ca^{2+} is replaced by Ba^{2+} in the external ringer solution, whereas the slow component of inactivation is largely unchanged (Lin et al. 2012). A plot of $I_{Ca(V)}$ inactivation following conditioning voltage pulses against membrane potential shows an inverse bell-shaped curve mirroring the I-V relationship which further suggests that inactivation is caused by Ca^{2+} influx (Forsythe et al. 1998). The Ca^{2+} sensor for Ca^{2+}-dependent inactivation must be pre-associated with the channel itself because, similarly to what is observed in dorsal root ganglion neurons (Morad et al. 1988), the onset of current activation after UV-flash photolysis of caged Ca^{2+} is virtually instantaneous at the calyx of Held (Fig. 9.5) (Lin et al. 2012).

Approximately 45 % of $I_{Ca(V)}$ inactivates during 100 ms step depolarizations. This fractional inactivation is remarkably insensitive to manipulations of the

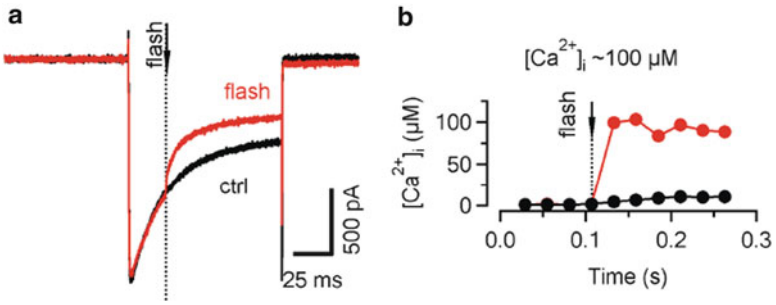

Fig. 9.5 Onset kinetics of Ca^{2+}-dependent inactivation after flash photolysis of caged Ca^{2+}. $I_{Ca(V)}$ (**a**) elicited by step depolarizations to 0 mV with (*red*) and without (*black*) a UV-light flash (*dotted line*) delivered ~25 ms after current onset. Corresponding changes in $[Ca^{2+}]_i$ are illustrated in (**b**). The post flash $[Ca^{2+}]_i$ increased to ~100 µM. Note the immediate acceleration of the inactivation time course after Ca^{2+} uncaging (Modified from Lin et al. 2012)

intracellular Ca^{2+} buffering strength as it is virtually unchanged when replacing 0.5 mM EGTA by 30 mM BAPTA in the pipette solution. If Ca^{2+} channels are clustered at the active zone (Roberts et al. 1990; Llinas et al. 1992; Westenbroek et al. 1995; DiGregorio et al. 1999; Harlow et al. 2001; Nagwaney et al. 2009), high concentrations of Ca^{2+} are also reached in between channels and the local $[Ca^{2+}]_i$ transient built-up by the overlapping Ca^{2+} domains within such channel clusters may be difficult to buffer effectively. This may explain why lowering the density of functional Ca^{2+} channels by application of the open-channel blocker ω-AgaTx quite effectively reduces the amount of inactivation during voltage steps. In contrast, reducing Ca^{2+} influx to a similar extent by means of lowering the external Ca^{2+} concentration does not affect inactivation significantly (Lin et al. 2012).

The $[Ca^{2+}]_i$ requirements for inducing Ca^{2+}-dependent inactivation can be directly assayed using flash photolysis-induced Ca^{2+} uncaging (Lin et al. 2012). Using such approach, the amount of $I_{Ca(V)}$ inactivation after step-like UV flash-induced elevations of $[Ca^{2+}]_i$ to various levels can be quantified. Inactivation starts to occur at elevations of $[Ca^{2+}]_i$ >2 µM and reaches a plateau at ~100 µM. Half-maximum inactivation is measured at ~6 µM. For comparison, a single presynaptic AP elevates $[Ca^{2+}]_i$ by only ~400 nM (Helmchen et al. 1997; Müller et al. 2007) but during repetitive AP firing at high-frequency the level of free intracellular Ca^{2+} can reach several micromoles (Billups and Forsythe 2002; Korogod et al. 2005; Müller et al. 2007). Thus, it is likely that Ca^{2+}-dependend inactivation in intact terminals is most effectively induced by AP trains (see below). Ca^{2+}-dependent inactivation of calyceal $I_{Ca(V)}$ depends on the interaction of the channels with calmodulin as it is attenuated by pre-incubating slices with the camodulin antagonist calmidazolium or intraterminal application of myosin light chain kinase (MLCK) peptide, a specific inhibitor of calmodulin (Xu and Wu 2005; Nakamura et al. 2008).

9.5 Recovery from Inactivation

When $I_{Ca(V)}$ inactivation is induced with very long depolarizations (1.9 s), complete recovery takes more than 1 min. The recovery has a bi-exponential time course with fast and slow time constants of ~7.5 s and 50 s, respectively, and the fast component accounting for about two thirds of the recovery (Forsythe et al. 1998). Presumably, after such prolonged periods of Ca^{2+} influx that are likely to saturate cytosolic Ca^{2+} buffers, the delayed clearance of Ca^{2+} from the cytoplasm may contribute to the very slow recovery from inactivation. After shorter voltage steps (100 ms), Ca^{2+} channels recover much more quickly from inactivation with fast and slow time constants of ~0.2 s and 5 s, respectively, and the fast component contributing about 75 % (Fig. 9.6) (Lin et al. 2012).

During step depolarizations under typical whole-cell recording conditions (0.5 mM EGTA), the global volume-averaged $[Ca^{2+}]_i$ can reach peak amplitudes of several micromoles and slowly decays back to its resting level with a bi-exponential time course. However, the time course of recovery from Ca^{2+} channel inactivation seems to be unrelated to the Ca^{2+} clearance time course but rather reflects intrinsic channel properties as it is unchanged even when $[Ca^{2+}]_i$ is strongly buffered by including 10 mM EGTA or BAPTA in the pipette solution, a manipulation which nearly completely suppresses the slowly decaying residual Ca^{2+} transient after step depolarizations (Fig. 9.6) (Lin et al. 2012). The slow recovery from inactivation allows accumulation of Ca^{2+} channel inactivation during repetitive channel gating such as during AP trains (see below).

9.6 Facilitation of Presynaptic VGCCs

Following pre-depolarizations, calcium channels gating is accelerated (Borst and Sakmann 1998a; Cuttle et al. 1998). Using paired-pulse protocols of short, 1 ms voltage steps to −10 mV, $I_{Ca(V)}$ is facilitated for inter-pulse intervals ≤100 ms. The facilitation is greater at shorter intervals with a maximum of about 20 % for intervals of 5–10 ms (Cuttle et al. 1998). The facilitation of $I_{Ca(V)}$ can be explained by an acceleration of the voltage-dependent rate constant (α_m) for opening which produces a hyperpolarizing shift in the I-V relationship of about −4 mV (Borst and Sakmann 1998a; Cuttle et al. 1998). The faster opening of the facilitated channel is particularly evident during steps to slightly more negative membrane potentials between −25 and 15 mV at which the activation rate of non-facilitated Ca^{2+} channels is relatively slow (Fig. 9.7).

It is conceivable that $I_{Ca(V)}$ may be tonically suppressed by presynaptic G protein-coupled receptors and relief from this suppression may cause facilitation. However, experiments using GTPγS and GDPβS rule out an involvement of G protein-coupled mechanisms (Cuttle et al. 1998). $I_{Ca(V)}$ facilitation is also unaffected by intense hyperpolarization following the conditioning pulse (Cuttle et al. 1998;

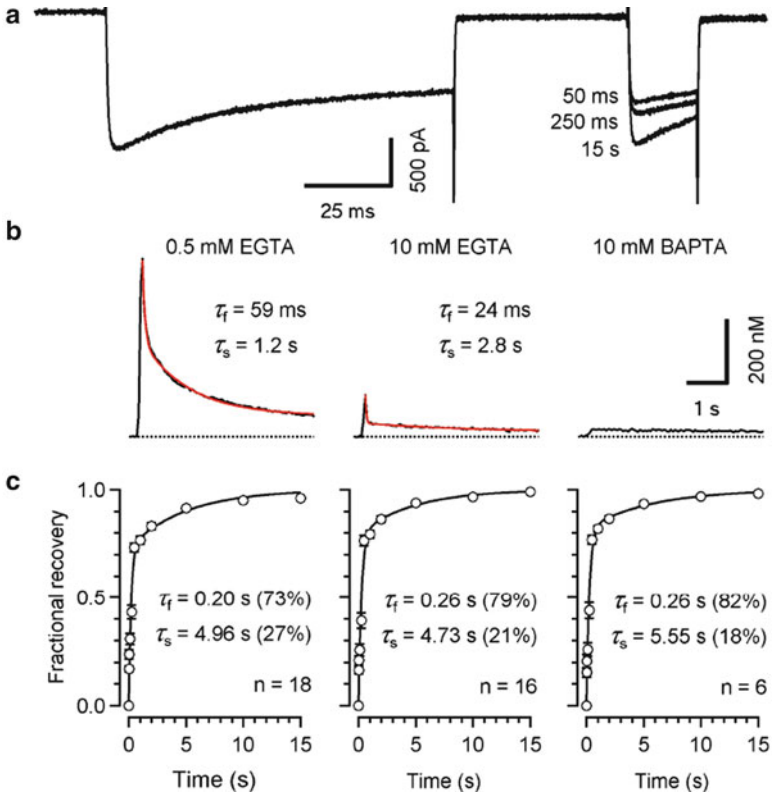

Fig. 9.6 Recovery of $I_{Ca(V)}$ from inactivation is slow. (**a**) Recovery of $I_{Ca(V)}$ from inactivation tested at variable intervals using a paired-pulse protocol consisting of a 100 ms depolarization to 0 mV followed by a 20 ms depolarization to 0 mV at variable inter-stimulus interval. Pipette solution contained 0.5 mM EGTA. Traces for three different recovery intervals are shown superimposed. (**b**, **c**) The time course of recovery from inactivation is biphasic and insensitive to changes in Ca^{2+} buffer strength. (**b**) $[Ca^{2+}]_i$ transients evoked by 100 ms step depolarizations. The rise of global $[Ca^{2+}]_i$ is nearly completely suppressed when adding 10 mM BAPTA to the pipette solution (*right panel*). (**c**) Average time course of recovery from inactivation. *Solid lines* represent double exponential fits. Fast and slow time constants were similar for the three $[Ca^{2+}]_i$ buffering conditions. Relative amplitudes of fast and slow time constants are given in *parenthesis* (Modified from Lin et al. 2012)

Lin et al. 2012), arguing against a contribution of voltage. Rather, it depends on the build-up of intracellular Ca^{2+}, because its magnitude is proportional to the Ca^{2+} influx (Borst and Sakmann 1998a; Cuttle et al. 1998) and it is abolished when substituting external Ca^{2+} with Ba^{2+} (Fig. 9.7a, b) (Cuttle et al. 1998; Lin et al. 2012). High concentrations ($\geq$10 mM) of the fast Ca^{2+} buffer BAPTA reduce the amount of facilitation of the peak $I_{Ca(V)}$ elicited by action potential waveforms (Borst and Sakmann 1998a) or short AP-like voltage steps whereas the slow buffer EGTA is much less effective (Cuttle et al. 1998). On the other hand,

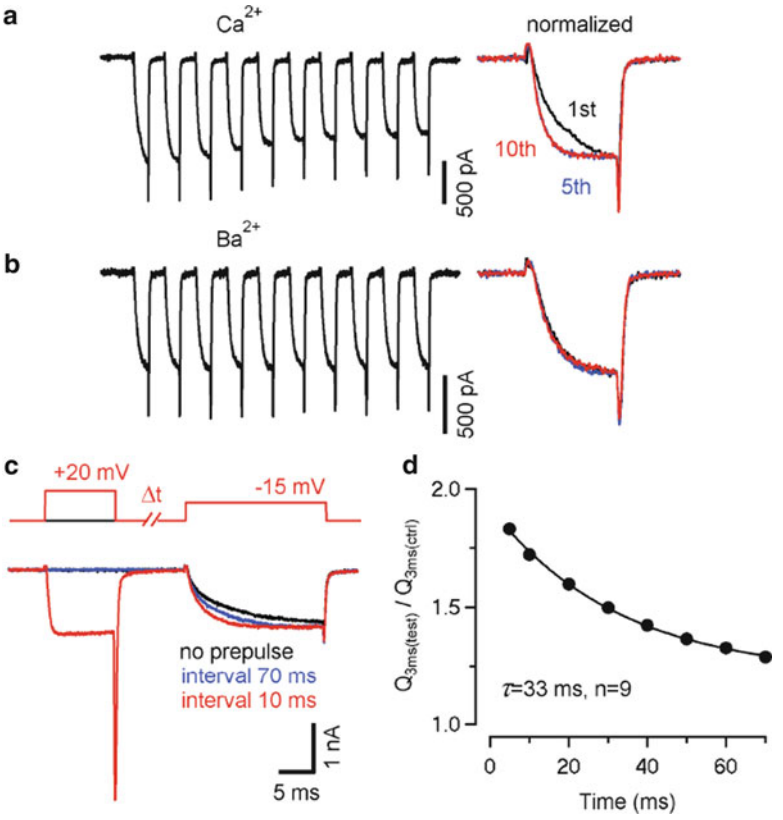

Fig. 9.7 Ca^{2+} dependent facilitation of $I_{Ca(V)}$ during pre-pulse protocols. (**a**, **b**) $I_{Ca(V)}$ elicited by a 100 Hz train of 5 ms steps from $V_h = -80$ mV to -20 mV in P8 calyces. (**a**) With Ca^{2+} as the charge carrier (the activation of $I_{Ca(V)}$ strongly accelerated from the first to the fifth voltage step and remained fast during later steps. (**b**) No change in activation kinetics was observed with external Ba^{2+}. *Right panel*: first, fifth and tenth $I_{Ca(V)}$ shown superimposed for comparison after normalizing to the same peak amplitude. (**c**) Sample traces of $I_{Ca(V)}$ without (*black trace*) or with prepulse at 10 ms (*red*) or 70 ms interval (*blue*). (**d**) The relaxation of $I_{Ca(V)}$ facilitation is an order of magnitude faster than recovery from inactivation. *Solid line* represent single exponential fit with a decay time constant as indicated (Modified from Lin et al. 2012)

when facilitation is assayed as increase in charge transfer during the initial 3 ms of $I_{Ca(V)}$ elicited by voltage steps to -20 or -15 mV, including either 10 mM EGTA or 10 mM BAPTA reduces facilitation only slightly (Lin et al. 2012). The relaxation of $I_{Ca(V)}$ facilitation follows an exponential time course with a time constant of ~30 ms (Fig. 9.7c, d) (Cuttle et al. 1998; Lin et al. 2012) and is thus about an order of magnitude faster than the recovery from current inactivation. Therefore, accumulation of Ca^{2+} channel facilitation during repetitive activation of $I_{Ca(V)}$ is more restricted and can only occur at very short inter-stimulus intervals.

UV-flash photolysis-evoked Ca^{2+} uncaging elicites $I_{Ca(V)}$ facilitation of similar magnitude compared to that evoked by voltage pre-pulses (Lin et al. 2012). In such experiments, elevation of $[Ca^{2+}]_i > 1$ μM are required to induce appreciable $I_{Ca(V)}$ facilitation and concentrations >20 μM do not further increase the amount of facilitation. However, the magnitude of facilitation is generally small and, especially at high Ca^{2+} concentrations, it is difficult to separate facilitation from inactivation which makes establishing a dose-response relationship between $[Ca^{2+}]_i$ and $I_{Ca(V)}$ facilitation more complicated. Nevertheless, it appears as if the $[Ca^{2+}]_i$ requirements for inducing $I_{Ca(V)}$ facilitation and inactivation are rather similar. The facilitation of Ca^{2+} currents seems to depend largely on an interaction with the high-affinity calcium-binding protein neuronal calcium sensor 1 (NCS-1). Direct loading of NCS-1 into calyx terminals mimics and partially occludes Ca^{2+}-dependent facilitation of $I_{Ca(V)}$ (Tsujimoto et al. 2002). On the other hand, $I_{Ca(V)}$ facilitation during trains of very high frequencies (500 Hz) is also sensitive to inhibition of calmodulin, suggesting an additional Ca^{2+} channel-calmodulin interaction (Nakamura et al. 2008).

9.7 Modulation by G Protein-Coupled Receptors

Calyx of Held synapses expresses presynaptic group II and III metabotropic glutamate receptors (mGluRs) that are negatively coupled to neurotransmitter release (Barnes-Davies and Forsythe 1995; Takahashi et al. 1996; von Gersdorff et al. 1997). In addition to mGluR agonists, $GABA_B$ receptor, α2-adrenoreceptor, 5-HT receptor and A_1 receptor agonists can presynaptically attenuate synaptic transmission (Barnes-Davies and Forsythe 1995; Leao and von Gersdorff 2002; Kimura et al. 2003; Mizutani et al. 2006). Baclofen, L-AP4, 5-HT, adenosine and noradrenaline inhibit the presynaptic $I_{Ca(V)}$ without affecting K^+ currents or AP-waveform, and their inhibitory effect on EPSCs can be fully explained by the reduction in Ca^{2+} influx (Takahashi et al. 1996, 1998; Leao and von Gersdorff 2002; Kimura et al. 2003; Mizutani et al. 2006). A_1 receptors share a common mechanism for the presynaptic inhibition with $GABA_B$ receptors because in the presence of baclofen, adenosine no longer attenuates EPSCs (Kimura et al. 2003). Similarly, L-AP4 occludes the inhibition by noradrenaline of EPSCs when applied first. However, mGluRs are more potent modulators of $I_{Ca(V)}$ because after inhibition by noradrenaline, L-AP4 is able to further inhibit the Ca^{2+} current (Leao and von Gersdorff 2002). In contrast to its inhibitory effect at the calyx of Held, noradrenaline potentiates the size of EPSCs at another calyx-type synapse in the chick ciliary ganglion and this is due to a cGMP-dependent mechanism that increases the Ca^{2+} sensitivity of exocytosis (Yawo 1999).

What are the endogenous agonists for the modulation of $I_{Ca(V)}$ by G protein-coupled receptors? Obviously, synaptically released glutamate can induce feedback inhibition via mGlu autoreceptor activation. In fact, mGluR antagonists attenuate steady state depression of EPSCs during train stimulation (von Gersdorff et al. 1997;

Iwasaki and Takahashi 2001). Endogenous adenosine seems to be co-released during presynaptic AP firing, because application of the A_1 antagonist CPT slightly but significantly increases the steady-state EPSC amplitude during 10 Hz trains (Kimura et al. 2003). The MNTB also receives adrenergic input (Jones and Friedman 1983; Wynne and Robertson 1996). However, the physiological function of this adrenergic innervation is not well understood. Alternatively and/or additionally, the ambient concentrations of GABA, glutamate, adenosine or noradrenalin may provide tonic inhibition of $I_{Ca(V)}$ (Cavelier et al. 2005; Glykys and Mody 2007).

9.8 Modulation of Presynaptic Ca^{2+} Influx During AP-Like Stimulus Trains

The regulation of AP-driven Ca^{2+} influx represents a powerful mechanism to modulate synaptic strength because transmitter release is highly nonlinearly related to the intraterminal Ca^{2+} concentration at the calyx of Held, and even minute changes in presynaptic Ca^{2+} influx can strongly influence release probability (Takahashi et al. 1996; Borst and Sakmann 1999). When $I_{Ca(V)}$ is elicited repetitively using brief AP-like depolarizations, the current amplitude can be augmented or reduced depending on the inter-stimulus interval and number of stimuli applied. The frequency dependence of this modulation has been studied in calyx (Taschenberger et al. 2002; Xu and Wu 2005; Nakamura et al. 2008) and endbulb (Lin et al. 2011) terminals by applying trains of depolarizations (typically 1 ms to 0 mV, Fig. 9.8). At the calyx of Held, $I_{Ca(V)}$ inactivation is observed at stimulus frequencies <20 Hz. Maximum current inactivation of about 15–20 % is observed at 5–10 Hz. At frequencies ≥50 Hz, $I_{Ca(V)}$ is initially enhanced (up to ∼20 % for a frequency of 200 Hz) but thereafter declines (Xu and Wu 2005; Nakamura et al. 2008; Lin et al. 2011). The facilitation of the Ca^{2+} current may therefore contribute to synaptic facilitation, or it may counterbalance the processes leading to synaptic depression at high presynaptic discharge rates. During low frequency firing, Ca^{2+} current inactivation contributes significantly to synaptic depression at the young calyx of Held synapse (Xu and Wu 2005). Surprisingly, $I_{Ca(V)}$ inactivation is completely absent during trains of depolarizations at endbulb terminals (Fig. 9.8). Whereas robust facilitation of $I_{Ca(V)}$ is observed at frequencies ≥20 Hz, $I_{Ca(V)}$ remained stable throughout the train during low-frequency stimulation (1–10 Hz) (Lin et al. 2011).

9.9 Developmental Refinements

During early postnatal development, endbulb (Neises et al. 1982; Bellingham et al. 1998; Limb and Ryugo 2000) as well as calyx (Kandler and Friauf 1993; Chuhma and Ohmori 1998; Taschenberger and von Gersdorff 2000; Iwasaki and Takahashi 2001; Joshi and Wang 2002; Taschenberger et al. 2002, 2005) synapses

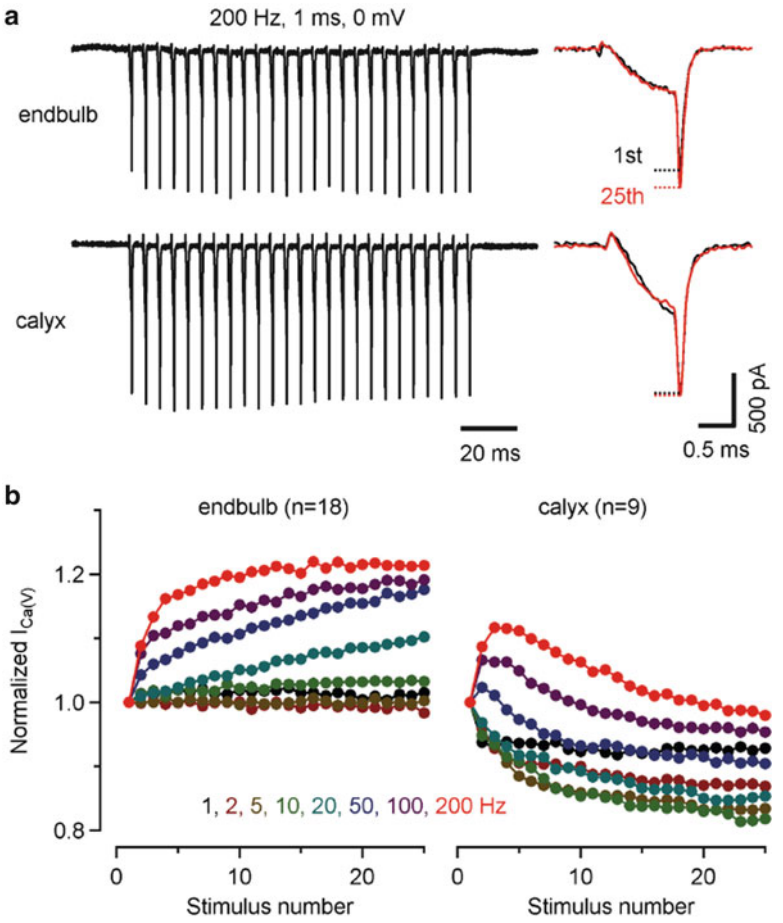

Fig. 9.8 Modulation of $I_{Ca(V)}$ during trains of AP-like depolarizations. (**a**) 200 Hz trains of $I_{Ca(V)}$ elicited by brief depolarizations (1 ms, 0 mV) recorded with a pipette solution containing 0.5 mM EGTA from an endbulb (*top*) and a calyx (*bottom*) terminal (*left panels*). Initial and final $I_{Ca(V)}$ are shown superimposed for comparison (*right panels*). Facilitation of $I_{Ca(V)}$ was observed during train stimulation of endbulb terminals. In calyx terminals, $I_{Ca(V)}$ inactivated after initial facilitation. (**b**) Modulation of presynaptic $I_{Ca(V)}$ during train stimulation at frequencies ranging from 1 to 200 Hz. Results from 18 endbulb (*left*) and nine calyx terminals (*right*) (Modified from Lin et al. 2011)

undergo several morphological and functional modifications which eventually transform them into fast and reliable relay synapses. Even though many of these developmental changes primarily affect the function of postsynaptic AMPA and NMDA receptor channels, a few prominent functional changes occur at the presynaptic site as well. Immature calyces express a mixture of N-, P/Q- and R-type VGCCs (Wu et al. 1998, 1999) whereas Ca^{2+} influx in more mature terminals predominantly depends on P/Q-type channels (Iwasaki and Takahashi 1998; Iwasaki et al. 2000).

It is possible that a similar developmental switch in the expression of α1-subunits occurs at endbulb terminals as well. However, $I_{Ca(V)}$ in P9–11 endbulb terminals is already exclusively generated by P/Q-type VGCCs (Lin et al. 2011). Interestingly, in P/Q-type channel k.o. mice, $I_{Ca(V)}$ is reduced by less than 50 % at the calyx of Held. The contribution of N-type channels to $I_{Ca(V)}$ increases nearly tenfold and thus largely compensates for the lack of the $Ca_V2.1$ subunit (Inchauspe et al. 2004; Ishikawa et al. 2005). In addition, the activation curve obtained from tail currents shows a depolarizing shift in $Ca_V2.1$-deficient compared to wildtype mice. This renders VGCCs less efficiently gated by the presynaptic AP waveform (Li et al. 2007) and, together with the lower Ca^{2+} channel density, is expected to cause a strongly reduced EPSC size. However, EPSC amplitudes in k.o. mice are surprisingly similar to those in wildtype mice (Ishikawa et al. 2005; Inchauspe et al. 2007). More importantly, facilitation of EPSCs, which is typically observed under low release probability conditions at wildtype synapses, is severely reduced in $Ca_V2.1$-deficient mice. This suggests that EPSCs facilitation is mediated to a large degree by Ca^{2+} current facilitation (Inchauspe et al. 2004; Ishikawa et al. 2005; but see Müller et al. 2008).

In addition to changes in the pharmacological profile of presynaptic VGCCs, a consequential change of the presynaptic AP waveforms occurs at the calyx of Held, with more mature calyx terminals having significantly briefer and faster APs (Fig. 9.9a) (Taschenberger and von Gersdorff 2000; Nakamura and Takahashi 2007). Because the number of VGCCs increases only moderately during development (Fig. 9.9b), the shortening of the AP waveform causes a significant reduction of the presynaptic AP-evoked $I_{Ca(V)}$ (Fedchyshyn and Wang 2005) and may thereby contribute to the observed reduction of the release probability in more mature calyx synapses (Iwasaki and Takahashi 2001; Taschenberger et al. 2002). Interestingly, the reduced Ca^{2+} influx per AP is partially compensated by a tighter spatial coupling between VGCCs and docked vesicles as suggested by the following two observations: (I) The slow Ca^{2+} chelator EGTA is more effective in reducing glutamate release when dialyzed into young compared to more mature terminals (Fedchyshyn and Wang 2005) indicating a developmental shortening of the diffusional distance between synaptic vesicles and Ca^{2+} channels. (II) Significantly higher peak $[Ca^{2+}]_i$ concentrations are required when modeling AP-evoked release transients of more mature synapses (Wang et al. 2008; Kochubey et al. 2009). Apparently, this spatial reorganization of channel-vesicle coupling relies on the $Ca_V2.1$ Ca^{2+} channel subunit. VGCCs and docked vesicles appears to be less tightly coupled in the absence of P/Q-type Ca^{2+} channels because after pre-incubation with the membrane permeable chelator EGTA-AM glutamate release is more strongly attenuated in $Ca_V2.1$-deficient compared to wildtype calyces (Inchauspe et al. 2007).

Immature calyx of Held terminals express a variety of G protein-coupled receptors (see above) some of which seem to be lost during synapse maturation. For example the inhibitory effects of 5-HT and adenosine are prominent in young terminals but become weaker during development, in parallel with a decrease in the A1 receptor immunoreactivity at the calyx terminal (Kimura et al. 2003; Mizutani et al. 2006). Similarly, the inhibition of EPSCs by mGluR agonists is strongly reduced in mature calyces (Renden et al. 2005). Moreover, the fraction

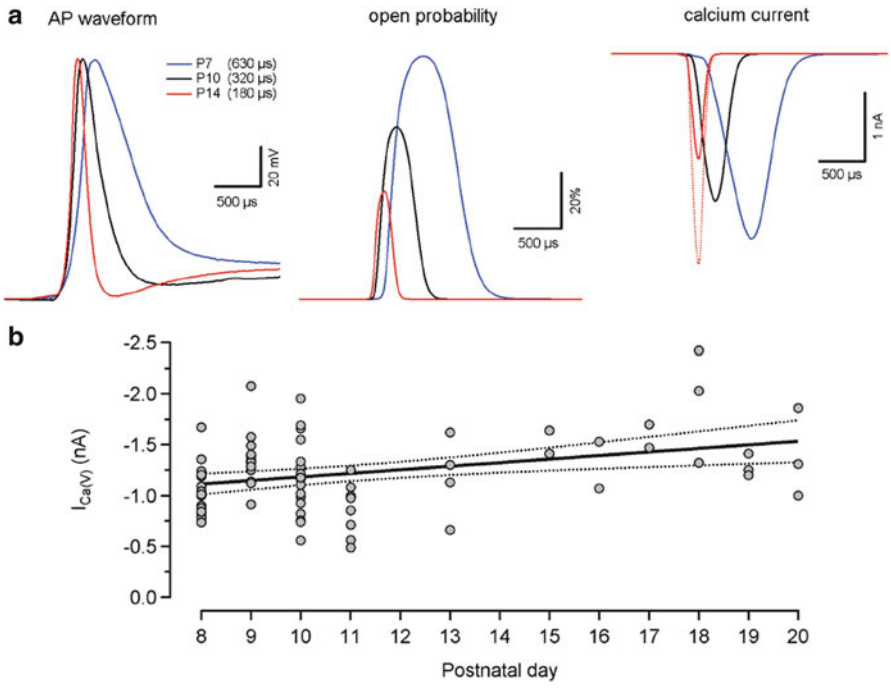

Fig. 9.9 Developmental changes in AP waveform and $I_{Ca(V)}$ at calyx terminals. (**a**) *Left*: Calyceal AP waveforms recorded at three different developmental stages. AP half-width is given in *parenthesis*. *Middle*: Corresponding time course of the activation parameter m^2 of a HH-model for $I_{Ca(V)}$ (Borst and Sakmann 1998b). Peak values were 88 %, 62 % and 39 % for the P7, P10 and P14 AP waveform, respectively. *Right*: Simulated AP-driven Ca^{2+} influx. Assuming equal Ca^{2+} channel densities at the three different ages, the predicted charge integrals were 1.4 pC, 0.6 pC and 0.2 pC. Note that even a doubling of the Ca^{2+} channel density at P14 (*red dotted line*) resulted in a significantly reduced AP-driven Ca^{2+} influx (0.4 pC) when compared to younger calyx terminals. (**b**) In mice calyces of Held, the amplitudes of $I_{Ca(V)}$ increase only slightly from P8 to P20. *Solid and dotted lines* represent linear regression and 95 % confidence intervals, respectively. The slope of the regression line is ~35 pA/day

of calyx synapses that are inhibited by noradrenaline strongly declines from 100 to ~25 % from P6 to P15, respectively, although the degree of inhibition does not change in responsive cells (Leao and von Gersdorff 2002). By contrast, activation of presynaptic $GABA_B$ receptors strongly inhibits the EPSCs throughout development (Renden et al. 2005).

Relatively little is known about the role of presynaptic afferent activity for the maturation of calyx synapses. Presynaptic firing activity and neurotransmitter release may regulate synaptic strength (Turrigiano et al. 1998; Murthy et al. 2001; Thiagarajan et al. 2005) by modulating expression, trafficking, degradation and function of synaptic proteins (Rao and Craig 1997; O'Brien et al. 1998; Ehlers 2000; Townsend et al. 2004). Whereas the down regulation of postsynaptic NMDA receptor channels is significantly delayed in deaf mice und thus seems to be

controlled by afferent nerve activity, presynaptic Ca^{2+} influx and the transmitter release machinery appeared to be relatively insensitive to deprivation from afferent nerve activity (Futai et al. 2001; Youssoufian et al. 2005; Erazo-Fischer et al. 2007). In $Ca_V1.3^{-/-}$ deaf mice, presynaptic $I_{Ca(V)}$ amplitudes were unaltered. The pharmacological profile of their presynaptic VGCCs was not tested, however Ca^{2+} current facilitation during high-frequency trains of AP-like depolarizations—which is the characteristic signature of calyceal P/Q-type VGCCs—is not different in deaf compared with wildtype mice.

9.10 Conclusions

Direct presynaptic patch-clamp recordings at endbulb and calyx synapses of the mammalian auditory brainstem have taught us valuable lessons about the physiology of presynaptic VGCCs at these terminals and these preparations will continue to be popular model synapses for examining the mechanisms of modulation of presynaptic Ca^{2+} influx. Much work is still ahead of us. A multitude of signaling molecules are involved in synaptic function and we have only begun to understand the machinery that orchestrates the molecular regulation of presynaptic Ca^{2+} channel function: presynaptic RIM scaffolding proteins are important for localizing Ca^{2+} channels to the active zone (Han et al. 2011) and the filamentous protein septin 5 participates in the developmental reorganization of VGCC to vesicle coupling (Yang et al. 2010). The fact that many conventional mouse knock out models are perinatally lethal has limited the usefulness of the calyx preparation for studying presynaptic function at the molecular level. However, Cre-lox based conditional knock out approaches (Han et al. 2011) and the use of viral vectors (Young and Neher 2009) may overcome these limitations in the future.

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Chapter 10
Use of Synthetic Ca^{2+} Channel Peptides to Study Presynaptic Function

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Abstract Small, synthetic peptides based on specific regions of voltage-gated Ca^{2+} channels (VGCCs) have been widely used to study Ca^{2+} channel function and have been instrumental in confirming the contribution of specific amino acid sequences to interactions with putative binding partners. In particular, peptides based on the Ca^{2+} channel Alpha Interaction Domain (AID) in the intracellular region connecting domains I and II (the I-II loop) and the SYNaptic PRotein INTeraction (synprint) site in the II-III loop have been widely used. Emerging evidence suggests that such peptides may themselves possess inherent functionality, a property that may be exploitable for future drug design. Here, we review our recent work using synthetic Ca^{2+} channel peptides based on sequences within the $Ca_V2.2$ amino terminal and I-II loop, originally identified as molecular determinates for G protein modulation, and their effects on VGCC function. These $Ca_V2.2$ peptides act as inhibitory modules to decrease Ca^{2+} influx with direct effects on VGCC gating, ultimately leading to a reduction of synaptic transmission. $Ca_V2.2$ peptides also attenuate G protein modulation of VGCCs. Amino acid substitutions generate $Ca_V2.2$ peptides with increased or decreased inhibitory effects suggesting that synthetic peptides can be used to further probe VGCC function and, potentially, form the basis for novel therapeutic development.

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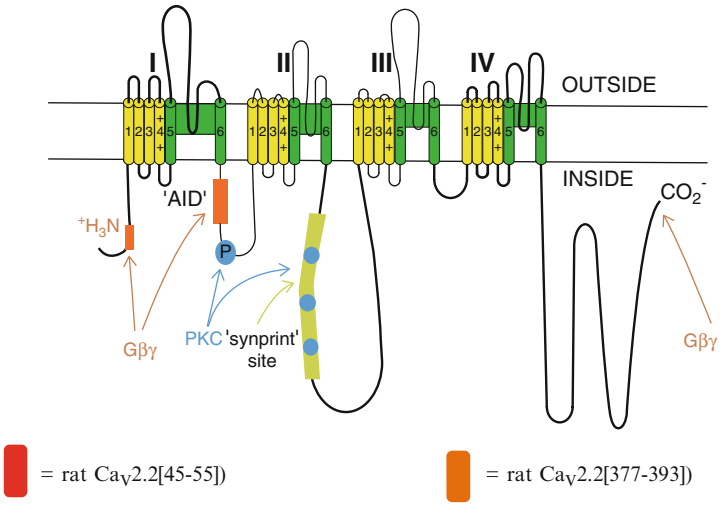
10.1 Introduction

The study of ion channel function has been greatly facilitated by the use of small, synthetic peptides. Such peptides are typically based on specific amino acid sequences shown to represent molecular determinants of ion channel function, often as identified via deletion constructs or site-directed mutagenesis studies. Peptides can either be designed to block exist interactions (loss-of-function) or to transfer functionality to a previously unresponsive protein (gain-of-function). Early examples of the latter include ion channel peptides based on the inactivation gate segment of voltage-dependent Na^+ channels (Eaholtz et al. 1994) and on the inactivation 'ball' particle in voltage-dependent K^+ channels (Zagotta et al. 1990; Stephens and Robertson 1995), both of which elements can confer inactivation properties onto a non-inactivating background. Synthetic Ca^{2+} channel peptides have been fundamental in advancing our understanding of VGCC function. In particular, peptides based on the AID region of the I-II loop regions have shed light both on G protein inhibitory mechanisms and on $Ca_V\beta$ function. In addition, peptides based on regions of the VGCC II-III loop implicated in interaction with synaptic proteins, the so-called synprint site, have revealed insights into synaptic protein function and presynaptic mechanisms, including vesicle function. This chapter reviews the use of synthetic Ca^{2+} channel peptides; including synprint peptides and, in particular, AID-based and amino terminal (NT) peptides initially implicated in G protein modulation, but which have also been shown to exert functional effects in their own right (Bucci et al. 2011). We restrict our review to peptides based on VGCC amino acid sequences and not those of interacting proteins that bind to the channel protein.

10.2 Synthetic $Ca_V2.2$ Peptides

10.2.1 Synthetic $Ca_V2.2$ Peptides Based on the 'Synprint' Site

A good deal of early work used a synprint peptide based on the $Ca_V2.2$[718-963] amino sequence of the II-III loop, the binding region for soluble NSF attachment protein receptor (SNARE) proteins, shown to interact with the synaptic core complex (containing syntaxin and the synaptosome-associated protein of 25 KDa (SNAP-25) bound to VAMP/synaptobrevin (Sheng et al. 1994; Rettig et al. 1996); Fig. 10.1). The synprint peptide was initially proposed to dissociate preformed complexes between syntaxin and rat brain $Ca_V2.2$ subunits (Mochida et al. 1996). Presynaptic injection of this peptide, or another $Ca_V2.2$[832–963] synprint peptide,



$Ca_V2.2$ amino terminal (NT) peptides	
NT peptide (rat $Ca_V2.2$[45-55])[1]:	YKQSIAQRART
NT peptide with substitutions to residues implicated in G protein modulation[1] and dominant-negative action on expression[2]:	YKQSIAQ**AA**AT
$Ca_V2.2$ I-II loop alpha-interaction domain (AID) peptides	
AID peptide (rat $Ca_V2.2$[377-393])[3,4]:	RQQQIERELNGYLEWIF
AID peptide with substitutions to residus implicated in G protein modulation[5]:	RQQQ**L**ERELNGYLEWIF
AID peptide with substitutions to residues implicated in $Ca_V\beta$ modulation[6]:	RQQQIERELNGYLE**A**IF

[1]Canti *et al.* (1999)
[2]Page *et al.* (2010)
[3]DeWaard *et al.* (1997)
[4]Zamponi *et al.* (1997)
[5]Herlitze *et al.* (1997)
[6]Berrou *et al.* (2002)

Fig. 10.1 Design of synthetic $Ca_V2.2$ peptides. Synthetic $Ca_V2.2$ peptides used in our studies were based on the $Ca_V2.2$[45–55] amino terminal 'NT peptide' and the $Ca_V2.2$[377–393] I–II loop alpha interaction domain 'AID peptide'. A double substitution to the NT peptide was made: R52A and R54A, these arginine residues were previously implicated in a range of Ca^{2+} channel functions. Substitutions to the AID peptide were I38L (within the QxxER Gβγ binding motif) and W391A, this tryptophan is conserved in all $Ca_V\alpha$ AID sequences and has been implicated in correct $Ca_V\beta$ function and aspects of G protein modulation

was shown to cause an inhibition of synaptic transmission between superior cervical ganglion neurons (SCGNs) in long-term culture (Mochida et al. 1996), pointing to a role for the region in exocytosis. Synprint peptides reduced fast, synchronous transmitter release, but increased asynchronous release and paired-pulse facilitation; these effects were reversible and occurred in the absence of effects on Ca^{2+} current

amplitude, voltage-dependent activation or steady-state inactivation (Mochida et al. 1996). Synprint peptide effects were attributed to an action whereby vesicles primed for synchronous release were shifted to a pathway less optimised for rapid, efficient exocytosis. The synprint peptide was further shown to block the voltage-dependent enhancement of transmitter release induced by a hypertonic solution of 0.5 M sucrose under conditions of tetanic stimulation in SCGNs (Mochida et al. 1998); blocking this voltage-dependent signal to the docking and release machinery was also proposed to contribute to the inhibition of synaptic transmission. Similarly, injection of a $Ca_V2.2$[718–963] synprint peptide into developing *Xenopus laevis* motor neurones inhibited synaptic transmission by reducing the Ca^{2+} sensitivity of neurotransmitter release, decreasing quantal content and increasing paired-pulse and tetanic facilitation (Rettig et al. 1997; Keith et al. 2007). The synprint peptide was also shown to bind to synaptotagmin, the Ca^{2+} sensor for transmitter release (Sheng et al. 1997), suggesting that synaptotagmin and syntaxin may compete for interaction with the synprint site. Thus, it was proposed that syntaxin may bind the synprint site to prevent the synaptic core complex from interacting with synaptotagmin; in order for efficient vesicular membrane fusion to occur it was further proposed that increased Ca^{2+} levels displace syntaxin in favour of synaptotagmin at the synprint site. Overall, evidence using synprint peptides suggest that prevention of syntaxin-$Ca_V2.2$ interaction causes a dissociation of Ca^{2+} channels from docked synaptic vesicles (Catterall 1999; Keith et al. 2007). Other studies have suggested that syntaxin binding enhances Ca^{2+} channel inactivation, which potentially would also act to inhibit transmitter release (Bezprozvanny et al. 1995). In this regard, the synprint peptide had functionally opposite effects to a mutant syntaxin, which lacks effects on Ca^{2+} channel gating (Bezprozvanny et al. 1995), at frog neuromuscular junctions (Keith et al. 2007). Here, synprint peptide effects could be reproduced by altering extracellular Ca^{2+} levels and it was proposed that, whilst synprint peptide major effects were on docked vesicles (as described above), they also act to relieve inhibition at those VGCCs not associated with docked vesicles.

Experiments using synprint peptides have also contributed to the demonstration that this region is a substrate for protein kinase C (PKC) and calmodulin-dependent protein kinase type II (CaM KII) phosphorylation and, moreover, that such phosphorylation inhibits peptide binding to syntaxin and SNAP-25 (Yokoyama et al. 1997). Use of deleted and amino acid substituted synprint peptides further identified two microdomains and specific residues within these regions involved in kinase regulation of $Ca_V2.2$ (PKC phosphorylation at serines 774 and 898 and CaMKII phosphorylation at serines 784 and 896); such data helped to formulate a structural model of the $Ca_V2.2$ synprint domain (Yokoyama et al. 2005). Interestingly, PKC phosphorylation did not dissociate $Ca_V2.2$ channel/syntaxin 1A complexes in recombinant full-length channels (Yokoyama et al. 2005); these data suggest that there needs to be some caution in extrapolating synthetic peptide effects in in vitro binding data to the situation in native channels where additional interactions may occur.

A further point of interest is that although Ca^{2+} channels in invertebrates such as *C. elegans*, *Drosophila*, and *Lymnaea* lack a synprint site, a synprint peptide was still able to cause a use-dependent inhibition of transmitter release in *Lymnaea* visceral dorsal 4 neurons (Spafford et al. 2003). These effects occurred in the absence of effects on Ca^{2+} current and may suggest that synprint peptides can inhibit transmitter release via interaction with other, as yet unknown binding partners. In this regard, the mammalian synprint region is known to interact with proteins additional to those of the synaptic core complex, such as regulators of G protein signaling (RGS), supporting evidence for this interaction came from the demonstration that synprint peptides inhibited RGS12 modulation of $Ca_V2.2$ (Richman et al. 2005).

More recently, synprint peptides have been introduced directly to immature calyx of Held presynaptic terminals and reported to have effects on endocytotic, rather than exocytotic, pathways at these synapses (Watanabe et al. 2010). A $Ca_V2.2$[832–963] synprint peptide was shown to cause an increase in membrane capacitance changes associated with synaptic vesicle exocytosis and intraterminal Ca^{2+} current amplitude, but to block membrane capacitance changes associated with endocytosis (Watanabe et al. 2010). This study showed that the μ subunit of AP-2, an adaptor protein for clathrin-mediated endocytosis, was also able bind to the synprint site and could compete with synaptotagmin-synprint interaction; the authors suggested that synprint peptides disrupted AP-2μ-synaptotagmin interaction to affect endocytosis (Watanabe et al. 2010). This study highlights the use of synthetic Ca^{2+} channel peptides as useful probes to investigate disparate pathways and distinct channel functions.

It is clear that the Ca_V2 II-III loop and, in particular, the synprint region, represents a hot-spot for protein-protein interactions and modulation; in the future, it will be of interest to use further defined synprint peptides to interfere selectively with specific interactions and probe Ca^{2+} channel function in more detail.

10.2.2 Synthetic $Ca_V2.2$ Peptides Based on Sites Involved in G Protein Modulation

Synthetic peptides have also been instrumental in defining regions involved in G protein modulation of VGCCs. Following the original identification of Gβγ subunits as the primary mediators of G protein-coupled receptor (GPCR) modulation of VGCCs (Ikeda 1996; Herlitze et al. 1996), three major Gβγ interaction sites were identified: (i) the amino terminal (NT), (ii) the I-II loop (Page et al. 1998; Stephens et al. 1998; Simen and Miller 1998, 2000; Canti et al. 1999), which contains the Ca_Vβ alpha interaction domain (AID) (Pragnell et al. 1994), and (iii) the carboxyl terminal (CT) (Qin et al. 1997; Li et al. 2004) (Fig. 10.1, reviewed by Dolphin 2003; Tedford and Zamponi 2006). Despite the identification and extensive mapping of Ca_V2/Gβγ molecular interaction sites, the relative contribution of each element to

presynaptic G protein modulation remains to be fully elucidated. Although it was initially believed that the I-II loop played the most prominent (or even exclusive) role (De Waard et al. 1997; Zamponi et al. 1997), subsequent studies have suggested that the I-II loop is not obligatory for G protein modulation (Qin et al. 1997; Stephens et al. 1998). Our earlier work showed that the NT contains specific determinants for G protein modulation of $Ca_V2.2$ and $Ca_V2.3$ subunits (Page et al. 1998; Stephens et al. 1998; Canti et al. 1999). Subsequently, Agler et al. (2005) confirmed the importance of the NT to Ca^{2+} channel function by demonstrating that this region can bind to the I-II loop. This study proposed that the NT domain may constitute a physical G protein-gated inhibitory module and indicates a more complicated interaction between different regions within the $G\beta\gamma/Ca_V2$ complex than was initially proposed. Overall, the CT is suggested to play a more minor role, although this region has been shown to increase $G\beta\gamma$ affinity for the channel (Li et al. 2004). Thus, we have used $Ca_V2.2$ peptides based on specific NT and AID amino acid sequences reported in the literature to represent binding sites for inhibitory G protein $\beta\gamma$ subunits in our work (Fig. 10.1).

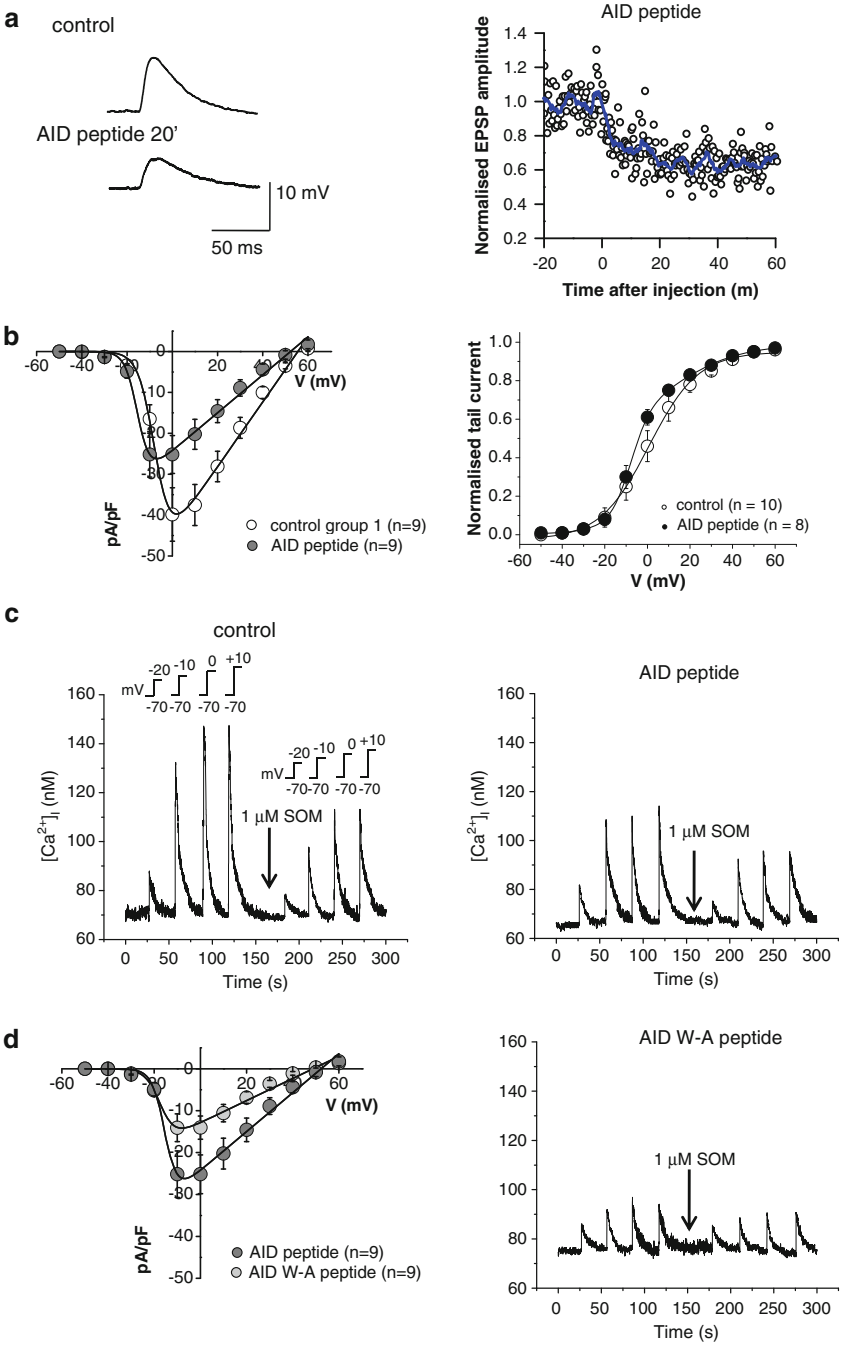
AID peptides based on Ca_V1 and Ca_V2 sequences, although possessing some amino acid differences, appear to be well conserved functionally (Van Petegem et al. 2008). AID-based peptides were important tools used to identify regions within the I-II loop as major sites of $Ca_V2/G\beta\gamma$ interaction (Herlitze et al. 1997; Zamponi et al. 1997; Furukawa et al. 1998; Bucci et al. 2011). Thus, AID based peptides blocked $G\beta\gamma$-mediated shifts in voltage dependence of activation curves and, also, prepulse facilitation of inhibited channels. The AID region is so named as it also mediates $Ca_V\alpha1/Ca_V\beta$ interaction and AID peptides have also been used to probe $Ca_V\beta$ function. AID peptides were original demonstrated to prevent the association of $Ca_V\beta$ subunits with $Ca_V\alpha_1$ I-II loop GST-fusion proteins in vitro (De Waard et al. 1995), thus implicating this region as an important high affinity $Ca_V\alpha_1/Ca_V\beta$ interaction site ($K_D \sim$10–30 nM, see Van Petegem et al. 2008). Initial experiments showed that AID peptides were unable to dissociate preformed $Ca_V\alpha_1/Ca_V\beta$ interaction in vitro (De Waard et al. 1995). By contrast, it was reported that an AID peptide could displace $Ca_V\beta$ from AID-affinity beads and cause a reduction in Ca^{2+} channel open probability (Hohaus et al. 2000). Moreover, an AID-based peptide containing flanking sequences was able to promote $Ca_V\beta$ dissociation from GST I-II loop fusion protein (Bichet et al. 2000). However, in the latter study effects of AID peptides were not replicated in native neurons, the lack of effect being attributed to the presence of additional $Ca_V\alpha_1/Ca_V\beta$ interaction sites. In these studies, AID peptide effects may also be explained by direct peptide binding to sites alternative to the AID in full-length native channels. AID peptides have also been co-purified with $Ca_V\beta$ and high resolution X-ray crystallographical analysis has revealed rich insights into $Ca_V\beta$ function (Opatowsky et al. 2004; Van Petegem et al. 2004, 2008). The AID region clearly represents an important integrative hotspot on the $Ca_V\alpha_1$ subunit, and AID peptides have also been used to block interactions between several other effectors, including binding of the small G-protein protein

Gem to $Ca_V\beta$ subunits (Sasaki et al. 2005) and, also, axonal collapsin response mediator protein 2 (CRMP2) to $Ca_V2.2$ (Wilson et al. 2012); the latter interaction was also inhibited by a $Ca_V1.2$ CT peptide.

By contrast to widely-used AID-based peptides, much less work on NT peptides has been performed. However, a NT peptide corresponding to the full-length $Ca_V2.2$[1–95] has been shown to cause a 'constitutive' inhibition of Ca^{2+} channel gating in the presence of $G\beta\gamma$ (Agler et al. 2005). In this study, it was demonstrated that the NT[1–95] peptide binds to the I-II loop in yeast two hybrid assays and that these region associate in FRET assays to promote this inhibitory interaction (Agler et al. 2005). Similarly, co-expression of this NT domain caused a dominant-negative suppression of Ca_V2 expression (Page et al. 2010); however, this study failed to reveal an interaction between NT[1–95] and, rather, proposed that the NT may interact with a alternative VGCC sites to cause functional effects. However, these data are consistent with inherent activity of Ca^{2+} channel peptides. More recently, an AID peptide and a $Ca_V1.2$ CT peptide were shown to reduce depolarization-induced Ca^{2+} influx in dorsal root ganglion (DRG) cells; moreover, the CT, but not the AID, peptide was able to inhibit depolarization-stimulated calcitonin gene-related peptide transmitter release in these cells (Wilson et al. 2012). Subsequent studies showed the CT peptide to possess anti-nociceptive effects in different models of neuropathy (Wilson et al. 2012). The mechanism of action of these peptides was attributed to inhibition of the interaction of CRMP2, a protein which stimulates VGCC activity, with its Ca^{2+} channel binding sites.

10.3 Use of Superior Cervical Ganglion Neurons (SCGNs) to Study Synthetic $Ca_V2.2$ Peptide Effects

We have used SCGNs as a model system to study $Ca_V2.2$ peptide actions. SCGNs maintained in long-term culture are a useful model to study presynaptic regulation of synaptic transmission, including GPCR-mediated presynaptic inhibition, whereby $G\beta\gamma$ subunits, liberated by receptor activation, bind to and inhibit presynaptic $Ca_V2.2$ channels to limit cholinergic neurotransmission (Stephens and Mochida 2005). In this regard, $Ca_V2.2$ (N-type) Ca^{2+} channels predominantly mediate acetylcholine release in this system (Mochida et al. 1995, 2003). SCGN nerve terminals are also accessible to peptides injected into the presynaptic partner in synaptically-coupled neuronal pairs (Ma and Mochida 2007), supporting the utility of this preparation for studying effects of $Ca_V2.2$ peptides on presynaptic mechanisms. In our experiments, we have used long-term SCGN cultures and, also, isolated cells to investigate the effects of $Ca_V2.2$[45–55] amino terminal 'NT peptide' and a $Ca_V2.2$[377–393] I–II loop alpha interaction domain 'AID peptide' (Fig. 10.1) on Ca^{2+} channel function in vitro.


a
control
AID peptide 20'
10 mV
50 ms
AID peptide
Normalised EPSP amplitude
Time after injection (m)
b
V (mV)
pA/pF
control group 1 (n=9)
AID peptide (n=9)
Normalised tail current
control (n = 10)
AID peptide (n = 8)
V (mV)
c
control
mV
-20 -10 0 +10
-70 -70 -70 -70
1 μM SOM
[Ca2+]i (nM)
Time (s)
AID peptide
d
AID peptide (n=9)
AID W-A peptide (n=9)
AID W-A peptide

10.3.1 Synthetic $Ca_V2.2$ Peptides Inhibit Synaptic Transmission

Our first clue that $Ca_V2.2$ peptides possess inherent functionality in our system came from experiments in which peptides were injected directly into the presynaptic partner of synaptically-coupled SCGNs. We initially demonstrated that either the NT or the AID peptide themselves caused a reduction in cholinergic transmission; for example, the effects of the AID peptide are shown in Fig. 10.2a. AID peptide effects were dose-related and were not seen for a scrambled peptide (Bucci et al. 2011). We previously showed that the $Ca_V2.2$ NT[44–55] sequence represents an important domain for G protein modulation (Canti et al. 1999). Following reports that a NT[1–95] peptide represents an inhibitory module (Agler et al. 2005; Page et al. 2010), we investigated whether a smaller NT[44–55] peptide may also have inhibitory effects. In support of our hypothesis, a similar inhibition of cholinergic transmission in SCGNs to that demonstrated for the AID peptide was seen for our NT peptide (Bucci et al. 2011). These data showed that a restricted $Ca_V2.2$ NT domain could instigate a form of inhibitory modulation.

10.3.2 Synthetic $Ca_V2.2$ Peptides Inhibit Ca^{2+} Channels by Affecting Activation Gating

Neurotransmitter release at presynaptic terminals is mediated by Ca^{2+} influx via VGCCs; at SCGNs, $Ca_V2.2$ (N-type) Ca^{2+} channels predominantly mediate acetylcholine release (Mochida et al. 1995, 2003). Therefore, we investigated the effects of $Ca_V2.2$ peptides on Ca^{2+} current. In these experiments, the situation at the SCGN presynapse was modelled using somatic recordings from isolated SCGNs. Introduction of either the NT or the AID peptide via a patch clamp electrode caused a clear decrease in whole-cell Ca^{2+} current; for example, the effects of the AID

Fig. 10.2 Effects of AID-based peptides in SCGNs. (**a**) Presynaptic injection of the AID peptide (1 mM) into SCGN synapses reduced EPSP amplitude; the inhibition of synaptic transmission is consistent with inherent functionality of synthetic $Ca_V2.2$ peptides. (**b**) Somatic injection of the AID peptide (1 mM) via a patch electrode caused reduction in whole cell Ca^{2+} current; normalized tail current amplitude was fitted with Boltzmann functions, current inhibition was accompanied by a shift from a control single Boltzmann distribution to a double Boltzmann distribution in the presence of synthetic $Ca_V2.2$ peptides. (**c**) Somatic injection of the AID peptide (1 mM) via a patch electrode caused reduction in depolarization-induced Ca^{2+} influx, note also the attenuation of G protein modulation (as induced by the GPCR agonist somatostatin (SOM)). Reductions in Ca^{2+} influx in (b) and (c) are consistent with direct effects of synthetic $Ca_V2.2$ peptides on VGCCs. (**d**) Somatic injection of the AID W-A peptide (1 mM) via a patch electrode caused reduction in whole cell Ca^{2+} current and in depolarization-induced Ca^{2+} influx. Effects of AID W391A peptide were greater than the parent AID peptide on these parameters, consistent with AID W-A peptide representing an improved inhibitory module

peptide are shown in Fig. 10.2b. Of interest here was that $Ca_V2.2$ peptide inhibition of Ca^{2+} influx was accompanied by effects on the voltage-dependence of activation of Ca^{2+} channels, effects which may explain this phenomenon. Thus, under control conditions, activation curves derived from tail current amplitudes could be fitted with a single Boltzmann distribution; in the presence of either the AID or the NT peptide, activation followed a dual Boltzmann distribution (Fig. 10.2b). A further point of interest is that inhibitory effects of Gβγ subunits on VGCC activation are similarly best described using a dual Boltzmann function (Bean 1989; Boland and Bean 1993). Therefore, our $Ca_V2.2$ peptides possess some overlapping functional characteristics with G protein modulation of Ca^{2+} channels; more specifically, these data are consistent with either the AID or the NT peptide being able to promote a constitutive inhibition in a manner analogous to Gβγ subunits. It is possible that direct peptide-induced changes to activation gating contribute to the reduction in Ca^{2+} current seen here. Consistent with peptide effects on Ca^{2+} current, we also found that $Ca_V2.2$ peptides reduced depolarization-induced Ca^{2+} influx, as measured by changes to intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels, in isolated SCGNs; for example, the effects of the AID peptide are shown in Fig. 10.2c. A similar reduction in depolarization-induced Ca^{2+} influx was reported for an AID and a CT peptide in DRG cells, this reduction was reflected by an attenuation of K^+-stimulated transmitter release in DRGs for the CT peptide, but was not seen for the AID peptide (Wilson et al. 2012). The differences in effects of similar AID peptides on transmitter release in DRGs in the Wilson et al. study and our study in SCGNs may reflect concentration differences between the systems. We have further demonstrated that AID peptides have effects on the action potential waveform in current-clamp recordings, causing a reduction in the after-hyperpolarization potential (Bucci et al. in preparation); such actions are characteristic of reduced Ca^{2+} influx through $Ca_V2.2$ channels in SCGNs (Davies et al. 1996; Vogl et al. 2012). Overall, these data are consistent with direct effects of $Ca_V2.2$ peptides on Ca^{2+} channels leading to a reduction of Ca^{2+} influx; at the presynapse, reductions in Ca^{2+} influx will translate to the demonstrated inhibition of transmitter release (see Sect. 10.3.5).

10.3.3 Synthetic $Ca_V2.2$ Peptides Attenuate G Protein Modulation of Ca^{2+} Channels

$Ca_V2.2$ peptides acted to attenuated hallmark characteristics of GPCR-mediated presynaptic inhibition. Thus, $Ca_V2.2$ peptides reduced noradrenaline- and somatostatin-induced inhibition of synaptic transmission, and negated the characteristic shift in mid-point of voltage-dependence of activation (Bucci et al. 2011, in preparation). As discussed above, previous studies using AID-based peptides have often attributed AID peptide action to a more straightforward competition for Gβγ binding site and subsequent ‘quenching’ of response. Our studies are also consistent with $Ca_V2.2$ peptides having inherent inhibitory effects on Ca^{2+} channel gating with such effects being accompanied by attenuated channel sensitivity

to G protein modulation. These data are not mutually exclusive with previous reports. Thus, we propose that $Ca_V2.2$ peptide action promotes a state which is unresponsive to G protein modulation, in agreement with lack of prepulse facilitation reported previously using AID peptides (Herlitze et al. 1997; Zamponi et al. 1997; Furukawa et al. 1998) and also seen in our studies. Such findings reconcile our data with those of previous reports and, overall, our data adds to evidence that $Ca_V2.2$ peptides may effectively block $G\alpha_{i/o}$ GPCR-mediated presynaptic inhibition.

10.3.4 Substitutions to Synthetic $Ca_V2.2$ Peptides Can Modify Their Inhibitory Effects

Given that synthetic Ca^{2+} channel peptides can possess inherent activity, it should then be possible to make substitutions to individual residues to probe their contribution to peptide function. With these points in mind, we made substitutions to those amino acids within the NT or AID peptide sequence that have previously been implicated as determinants of Ca^{2+} channel function (Fig. 10.1). The $Ca_V2.2$[44–55] NT region contains two arginine residues (R52 and R54) which have been widely implicated in a range of Ca^{2+} channel functions, including G protein modulation (Canti et al. 1999), $Ca_V\beta$-mediated inactivation properties (Stephens et al. 2000) and dominant-negative suppression of VGCC expression (Page et al. 2010). An NT peptide containing equivalent R52A,R54A mutations (NT R52A,R54A peptide) had no inhibitory effect on Ca^{2+} amplitude, activation gating or transmitter release in SCGNs (Bucci et al. 2011). By contrast, a $Ca_V2.2$[377–393] AID peptide with an isoleucine to leucine substitution at a position equivalent to I381 (within a proposed QxxER $G\beta\gamma$ binding motif, Dolphin 2003), which was shown to interfere with G protein modulation of the full length $Ca_V2.2$ channel (Herlitze et al. 1997), was found to fully retain inhibitory properties (Bucci et al. 2011). We have extended these studies to investigate an AID peptide with a tryptophan to alanine substitution at a position equivalent to $Ca_V2.2$ W391 ('AID W-A peptide') (Fig. 10.1). This tryptophan residue was of interest as it is conserved in all $Ca_V\alpha$ AID sequences (Pragnell et al. 1994) and has been widely reported to be an important determinant for $Ca_V\beta$ binding to the AID, representing a key determinant within the 'alpha-binding pocket' (Van Petegem et al. 2004, 2008). Moreover, $Ca_V2.2$ W391 was show to be vital for functional $Ca_V\beta$ effects on $Ca_V\alpha$, including modulation of Ca^{2+} current (Berrou et al. 2002) and, also, for aspects of G protein modulation (Leroy et al. 2005). Of interest here was that the AID W-A peptide showed increased inhibitory effects on Ca^{2+} channel function compared to the parent AID peptide. Thus, the AID W-A peptide showed stronger inhibition of several parameters including synaptic transmission and Ca^{2+} channel amplitude and, also, depolarization-induced changes in $[Ca^{2+}]_i$ and after-hyperpolarization potential (Fig. 10.2d). Overall, studies with the AID W-A peptide suggest that synthetic $Ca_V2.2$ peptides represent customisable modules which can be further optimised in vitro to produce more effective inhibitory agents.

10.3.5 Working Model for $Ca_V2.2$ Peptide Action

We propose a model whereby the $Ca_V2.2$[44–55] NT region and the $Ca_V2.2$ [377–393] AID can interact with the $Ca_V2.2$ subunit to inhibit VGCC function (Fig. 10.3). An important advance in theories regarding G protein inhibition was made by Agler et al. (2005), who used FRET measurements to demonstrate a physical *interaction* between the NT[1–95] peptide and the isolated I-II loop. By extension of this hypothesis, it may be suggested that the AID peptide could interact with the corresponding NT region of $Ca_V2.2$ (and vice versa for the NT peptide interacting with the AID region) to cause a similar inhibition in native, full length Ca^{2+} channels. Alternatively, $Ca_V2.2$ peptides may contribute to, or stabilise, a more complex, multi-element binding pocket suggested by Page et al. (2010). In support of a model involving interaction between the NT and the AID in native channels, we found that co-application of the AID and NT peptide negated the inhibitory effects shown by individual peptides on synaptic transmission and Ca^{2+} channel modulation and, also, G protein modulation (Bucci et al. 2011). Such an antagonism of effects may be explained by AID and NT peptides binding to each other to reduce inhibitory effects on the channel. We further propose that $Ca_V2.2$ peptide interaction leads to inhibitory effects on Ca^{2+} channel gating, manifest as a decrease in Ca^{2+} influx. Direct reductions in Ca^{2+} current seen here are supported by similar $Ca_V2.2$ peptide-mediated reductions in depolarization-induced Ca^{2+} influx in DRG cells (Wilson et al. 2012), and may also be consistent with reports that an AID peptide reduces open probability of Ca^{2+} channels (Hohaus et al. 2000).

Our proposal of an interaction between exogenous $Ca_V2.2$ peptides and the intact $Ca_V2.2$, suggests that the form of inhibition described here may be open to modulation by customised $Ca_V2.2$ peptides. In further support of this hypothesis, we demonstrate that the W-A substitution within the AID sequence resulted in a peptide with stronger inhibitory properties. We propose that the substitution of tryptophan, an amino acid with relatively bulky side chain, to a more compact alanine residue may improve access of the AID W-A peptide to its $Ca_V2.2$ binding site in comparison to the parent AID peptide. In this regard, structural studies predict that AID peptides are disordered and lack helical structure (Opatowsky et al. 2004; Van Petegem et al. 2008). Van Petegem and co-workers predict that binding affinity of residues within the AID sequence is dictated by the nature of individual side chain interactions. Thus, the presence of the alanine residue in the AID W-A peptide may modify channel flexibility required to transduce voltage and/or G proteins signals to the rest of the structure, resulting in increased channel inhibition. The model proposed by Agler et al. (2005) suggests that binding of the NT[1–95] peptide to the I-II loop promotes $G\beta\gamma$ binding, which remains bound during depolarization and, consequentially, is not subject to prepulse-mediated relief of G protein inhibition, as seen for the NT and AID peptide in our studies. In support of this hypothesis, we have used co-immunoprecipitation studies to show that $G\beta_1$ binding to $Ca_V2.2$ is increased in the presence of the AID peptide (Bucci et al. in preparation). However,

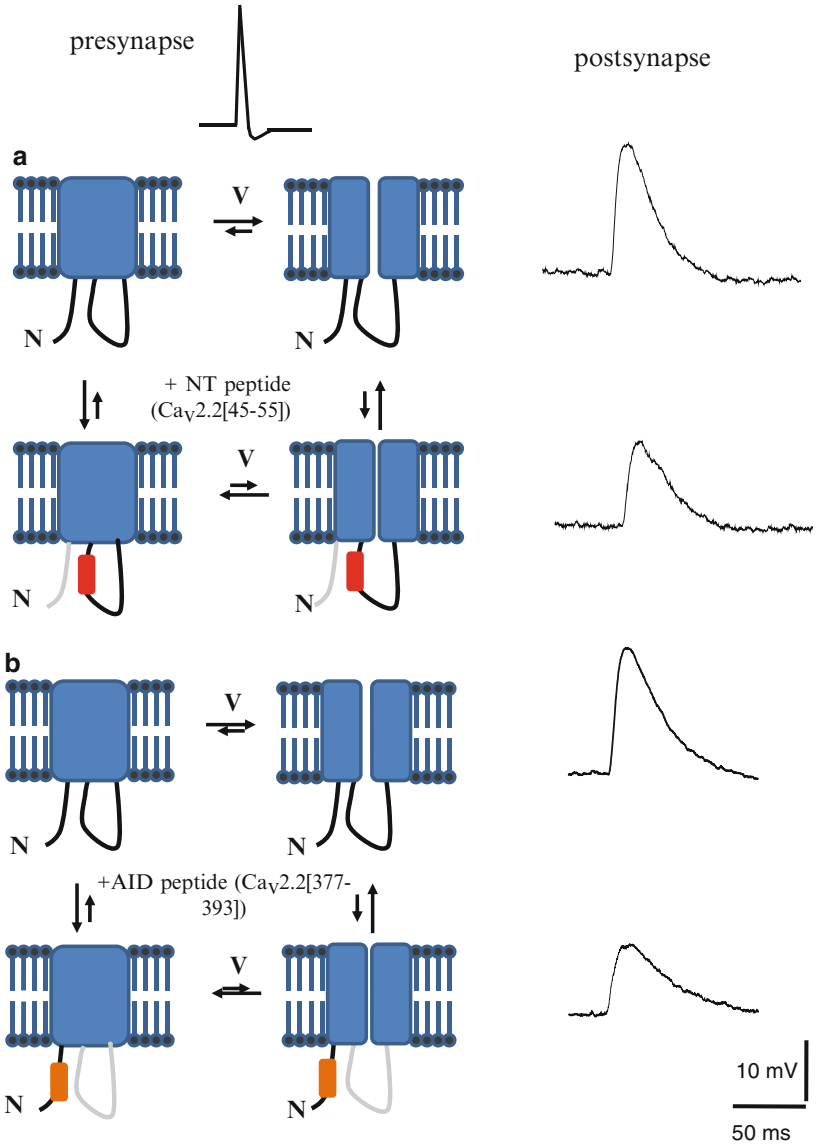


Fig. 10.3 Model of $Ca_V2.2$ peptide action. Proposed actions of the $Ca_V2.2$[45–55] amino terminal NT peptide (**a**) and the $Ca_V2.2$[377–393] I–II loop alpha interaction domain AID peptide (**b**). We propose that the NT peptide can interact with the VGCC, potentially via the AID, to inhibit VGCC gating; in SCGN synapses, VGCC inhibition reduces Ca^{2+} influx in response to presynaptic action potentials, causing a reduction in transmitter release as assayed by EPSP measurement. In a similar manner, we propose that the AID peptide can bind to the VGCC, potentially via the NT, and cause presynaptic inhibition in an analogous manner to that described for the NT peptide

the increased inhibition seen with the AID W-A peptide occurred independently of any increase in $G\beta_1$ binding. Overall, we suggest that our $Ca_V2.2$ peptides can insert into a binding site on the channel and inhibition is manifest in reduced Ca^{2+} influx due to changes in activation gating; in this configuration, G protein modulation is attenuated. Operation of such scheme (Fig. 10.3) at the presynapse, would result in inhibition of transmitter release as a consequence of reduced Ca^{2+} influx.

10.3.6 Conclusions

In our experiments, we have investigated effects of synthetic $Ca_V2.2$ peptides on synaptic transmission in synaptically-coupled SCGN synapses and examined the hypothesis that the NT and AID peptide act via direct inhibition of Ca_V2 channels using somatic electrophysiological recordings, calcium imaging and co-immunoprecipitation studies. We demonstrate that $Ca_V2.2$ peptides represent inhibitory modules in their own right and show that substitutions associated with changes to Ca^{2+} channel function have both positive and negative effects on peptide-induced inhibition. Although AID peptides were originally designed to bind to $G\beta\gamma$ and/or $Ca_V\beta$ and effectively remove their functionality, our more recent results clearly show that AID peptides possess their own inherent activity. Whilst $Ca_V2.2$ peptides such as the AID peptide have potential to bind to $G\beta\gamma$, we have found that the AID W-A peptide does not increase $G\beta$ recruitment to the $Ca_V2.2$ channels (Bucci et al. in preparation). The AID peptide may also bind to $Ca_V\beta$ subunits (Bichet et al. 2000); however, we also found that AID or AID W-A peptides had no effect on $Ca_V\beta$-$Ca_V2.2$ interaction (Bucci et al. in preparation). Together, these findings suggest that the increased inhibitory effects of AID W-A peptide are not associated with changes to G protein modulation or any dissociation of $Ca_V\beta$; rather, increased effects may reflect improved access to a peptide binding site on the channel and/or changes to inhibitory signal transduction. Overall, the demonstration of inherent functionality for our NT and AID peptide, and improved functionality of mutant peptides, suggests that we can design synthetic Ca^{2+} channel peptides to probe VGCC-mediated presynaptic mechanisms and, potentially, that such peptides may inform the design of therapeutic small molecules, as discussed more fully below.

10.4 Future Perspectives

Inhibition of Ca^{2+} influx by synthetic $Ca_V2.2$ peptide has been demonstrated in native neurons for NT, AID and, also, CT peptides (as described above and Bucci et al. 2011; Wilson et al. 2012). Such reports implicate synthetic $Ca_V2.2$ peptide as novel therapeutic agents acting at VGCCs. In this regard, an emerging concept is that Ca^{2+} channels may be targeted in a number of diseases, in particular in

the unmet clinical need associated with chronic and neuropathic pain (Vanegas and Schaible 2000; Zamponi et al. 2009; Park and Luo 2010). In general, presynaptic Ca_V2 subunits have received the most prominent attention, with the $Ca_V2.2$ (N-type) subunit representing a major pain target (Chaplan et al. 1994; Matthews and Dickenson 2001; Saegusa et al. 2001). This emphasis is best illustrated by the introduction of the analgesic ziconotide, a synthetic $Ca_V2.2$-blocking drug based on a peptide toxin isolated from the *Conus* sea-snail (Staats et al. 2004). Although establishing an important proof-of-concept, ziconotide has a narrow therapeutic window, a poor side-effect profile and must be delivered intrathecally; together, these issues have limited ziconotide's therapeutic impact. The studies described herein suggest that synthetic $Ca_V2.2$ peptides can form inhibitory molecules with potential to block $Ca_V2.2$ subunits expressed in pain pathways in vivo. A recent study has supported these aims; thus, a synthetic $Ca_V1.2$ CT peptide was shown to possess anti-nociceptive ability, attenuating AIDS therapy- and tibial nerve injury-induced periphery neuropathy (Wilson et al. 2012). This study also addressed a major issue regarding use of intracellularly acting peptides in therapeutic drug design, namely, can such peptide achieve sufficient efficacy; thus, the CT peptide was fused with the protein transduction domain of the HIV TAT protein to achieve cell penetration following intra-peritoneal injection. Wilson and co-workers have attributed $Ca_V2.2$ peptide effects to a loss-of-function dissociation of the binding partner, CRMP2, from the channel. Whether via direct or indirect action on the Ca^{2+} channel complex, the use of $Ca_V2.2$ Ca^{2+} channels holds therapeutic potential. The use of such synthetic peptides, and manipulation of their specific sequences, promises to provide tools to selectively target mechanisms of Ca^{2+} channel function and has potential to generate therapeutic agents. In summary, the use of synthetic Ca^{2+} peptides has value in increasing basic knowledge regarding mechanisms of $Ca_V2.2$ inhibition and, thus, may progress the therapeutic development of more efficacious, better tolerated small molecule entities, for example, in the pain field.

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Part IV
Calcium Channel Therapeutics

Chapter 11
Impact of a Loss-of-Function P/Q Type Ca^{2+} Channel Mutation on Excitatory Synaptic Control of Cerebellar Purkinje Neurons

David D. Friel

Abstract Voltage-gated Ca^{2+} channels are expressed throughout the central nervous system, where they regulate Ca^{2+} entry in excitatory and inhibitory neurons, pre- and postsynaptic cells, and do so in distinct subcellular domains, including synaptic terminals, dendrites and cell bodies. Consequently, mutations in genes encoding these channels have the potential to generate complex phenotypes involving functional changes in multiple cell populations that impact the way cells interact in neural circuits. An excellent illustration of this concept is provided by a loss-of-function mutation (called *leaner*) in the gene encoding the pore-forming subunit of P/Q type Ca^{2+} channels, which serves as a mouse model of Episodic Ataxia Type 2 (EA2), a heritable human P/Q Ca^{2+} channelopathy. Previous work has shown that the *leaner* mutation modifies intrinsic membrane properties of cerebellar Purkinje cells (PCs), which play a key role in cerebellar motor control. This review describes the effects of the mutation on excitatory synaptic inputs to PCs. Even though the *leaner* mutation dramatically changes excitatory postsynaptic currents that can be measured in voltage clamped PCs in acute cerebellar slices, there is surprisingly little effect on evoked excitatory postsynaptic *potentials*. This can be explained, at least in part, by effects of the mutation on intrinsic membrane properties of PCs that reduce the impact of mutation-induced changes in synaptic currents. These results exemplify the multifaceted nature of cell and circuit-level defects resulting from neuronal Ca^{2+} channelopathies.

Keywords P/Q channels • *CACNA1A* • $Ca_v2.1$ • Ca^{2+} channelopathies • Cerebellar ataxia • Excitatory synaptic transmission • Purkinje neurons • Synaptic currents • Parallel fibers • Climbing fibers • Complex spikes

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11.1 Introduction

While some genes are expressed mainly in a single cell type and perform a unitary function (e.g. genes encoding the subunits of hemoglobin, which are expressed mainly in red blood cells and regulate binding of gas molecules), other genes are expressed in multiple cell types and play functional roles that depend critically on cellular context. Such functional diversity complicates attempts to understand the biological role of expressed proteins, and the phenotypes that result from gene mutations, because we must consider not only the functional properties of the expressed proteins, but the impact of protein function in different cellular contexts. Since a given gene product may regulate different processes in different cells that communicate in different ways within cellular networks, understanding the ultimate physiological effects of a mutation may require an understanding of the functional consequences of the mutation at molecular, cellular and network levels.

Neuronal voltage-gated Ca^{2+} channels provide a prototypic example in which a multilevel approach is required to understand the biological impact of channel function in health and genetic disease. Voltage-gated Ca^{2+} entry serves in both voltage and chemical signaling to regulate many neuronal processes, including action potential firing, neurotransmitter release and gene expression (Catterall 2011). The specific effects of Ca^{2+} entry depend on cellular context. For example, Ca^{2+} entry in presynaptic terminals regulates neurotransmitter release, while in the neuronal cell body it regulates membrane excitability. Moreover, presynaptic Ca^{2+} entry in glutamate-containing neurons mediates postsynaptic excitation, while in gabaergic neurons it leads to postsynaptic inhibition. Therefore, mutations in a gene encoding a particular type of Ca^{2+} channel may have multiple functional consequences that depend on the subcellular distribution of the channel (e.g. presynaptic, dendritic, somatic), the postsynaptic effect of cell excitation (excitatory, inhibitory), and the pattern of synaptic communication between cells in neural circuits. One approach to understanding the ultimate consequences of such mutations is to characterize their effects at different organizational levels. For example, one might consider effects at the cellular level while synaptic interactions between neurons are blocked, and then at the circuit level while synaptic interactions are enabled, with the goal of ultimately addressing how these effects collectively account for phenotype at the level of the awake behaving animal. Such an approach has the potential to provide mechanistic insights into normal function, and to identify sites for effective therapeutic intervention in genetic disease.

11.1.1 P/Q Type Ca^{2+} Channels and Cerebellar Motor Control

An excellent example of a multifunctional Ca^{2+} channel is provided by P/Q type voltage-gated Ca^{2+} channels. P- and Q-type Ca^{2+} channels are different splice variants of the same channel transcript (Bourinet et al. 1999) and are expressed throughout the nervous system in a time- and cell type-specific manner (Hillman et al. 1991; Stea et al. 1994; Volsen et al. 1995; Westenbroek et al. 1995).

These channels control Ca^{2+} entry that triggers neurotransmitter release and regulates membrane excitability. Mutations in the human gene (*CACNA1A*) encoding the pore-forming α_1 subunit ($Ca_v2.1$) of P/Q channels cause Familial Hemiplegic Migraine Type-1 (FHM1), Spinocerebellar Ataxia Type 6 (SCA6) and Episodic Ataxia Type 2 (EA2) (Pietrobon 2010). These genetic diseases, which share in common cerebellar dysfunction, are clinically important, but treatments are limited, mainly because of an incomplete understanding of the causal link between the mutations and the neurological defects they produce. Since P/Q channels provide the dominant pathway for regulating voltage-sensitive Ca^{2+} entry in cerebellar Purkinje cells (PCs) (Mintz et al. 1992), and because PCs play a critical role in processing information in the cerebellar cortex, these cells have been one focus of study in research aimed at understanding why P/Q channel mutations disrupt cerebellar motor control (Pietrobon 2010; Rajakulendran et al. 2012).

11.1.2 Mouse Models of P/Q Ca^{2+} Channelopathies

One approach to studying how P/Q channels contribute to normal cerebellar function, and how P/Q channel mutations lead to cerebellar dysfunction, is to study the impact of mutations in the P/Q channel gene (*Cacna1a*) in mice. Loss-of-function (LOF) *Cacna1a* mutations cause ataxia in a way that implicates defects in cerebellar motor control (Sidman 1965; Victor et al. 2001; Pietrobon 2005). There are several LOF P/Q channel mutant mouse strains that display ataxia with different degrees of severity, including (from least to most severe): *rocker* (*rkr*) (Zwingman et al. 2001), *tottering* (*tg*) (Green and Sidman 1962), *rolling* Nagoya (tg^{rol}) (Oda 1973) and *leaner* (tg^{la}) (Meier and MacPike 1971); for reviews see (Liu et al. 2003; Pietrobon 2005). These mutations have been mapped and the corresponding changes in amino acid sequence in the expressed protein have been determined. Voltage clamp studies of dissociated PCs have shown that these mutations all reduce whole-cell Ca^{2+} channel current density, to an extent that parallels the severity of ataxia, with reductions of 23 % (*rocker*), ~24 % (*rolling* Nagoya), ~40 % (*tottering*) and ~60 % (*leaner*): (Dove et al. 1998; Lorenzon et al. 1998; Wakamori et al. 1998; Mori et al. 2000; Kodama et al. 2006). This parallel suggests that reduced Ca^{2+} entry through P/Q type Ca^{2+} channels is an important factor linking LOF channel mutations to disruptions of cerebellar motor control. Human P/Q channel mutations associated with EA2 are also associated with loss of channel function (Guida et al. 2001; Spacey et al. 2004; Pietrobon 2010; Rajakulendran et al. 2012).

11.1.3 Effects of P/Q Channel Mutations on Intrinsic PC Excitability

A number of studies have focused on the role of P/Q channels in regulating neurotransmitter release (Regehr and Mintz 1994; Mintz et al. 1995; Wheeler et al. 1996).

However, there is also abundant evidence indicating a role for these channels in defining intrinsic electrical properties of neurons. For example, PCs (both enzymatically dissociated and in acute cerebellar slices) spontaneously fire action potentials under the control of their intrinsic membrane properties (Llinas and Sugimori 1980a, b; Raman and Bean 1999; Womack and Khodakhah 2002). Ca^{2+} entry through P/Q type Ca^{2+} channels is necessary for this activity (in slices, but not in dissociated cells) since the activity is abolished by ωAgaIVA (Raman and Bean 1999; Womack and Khodakhah 2002), a specific blocker of P/Q channels. P/Q channels are also important in defining how PCs respond to electrical stimulation. For example, after suppressing spontaneous activity with steady injection of hyperpolarizing current, PCs respond to depolarizing current pulses with Na^+ and Ca^{2+} spikes in a manner that is graded with stimulus intensity (Llinas and Sugimori 1980a, b; Edgerton and Reinhart 2003; Ovsepian and Friel 2008). After blocking P/Q Ca^{2+} channels with ωAgaIVA, responses to such stimuli are strongly modified. For example, Ca^{2+} spikes can no longer be elicited, Na^+ spiking is not sustained (indicating enhanced Na^+ spike accommodation) and the slow voltage changes occurring during the interspike interval are shifted to more depolarized levels (Edgerton and Reinhart 2003; Ovsepian and Friel 2008). These changes can be explained based on what is known about P/Q channels and their role in PC excitability. Ca^{2+} spikes in these neurons depend on P/Q channels (Llinas and Sugimori 1980a, b; Edgerton and Reinhart 2003), and Ca^{2+} entry through these channels controls the activity of other channels, including Ca^{2+}-activated K^+ channels whose opening promotes membrane hyperpolarization (Llinas and Sugimori 1980a, b; Edgerton and Reinhart 2003; Womack et al. 2004). Thus, blocking P/Q channels in PCs would be expected to abolish Ca^{2+} spikes, and promote membrane depolarization, contributing to the observed depolarizing voltage shift during the interspike interval that presumably underlies increased Na^+ spike accommodation.

The effects of ωAgaIVA on evoked spiking indicate how an acute reduction in voltage-sensitive Ca^{2+} entry through P/Q channels can modify PC excitability. These effects can be compared to observations in PCs from LOF P/Q channel mutant mice, making it possible to address whether defects in excitability in mutant PCs are simply consequences of a reduction in ongoing voltage-sensitive Ca^{2+} entry. Indeed, in their responses to electrical stimulation, PCs from *leaner* mice show, at least qualitatively, each of the modifications of excitability observed in ωAgaIVA-treated wild type (WT) cells (Ovsepian and Friel 2008). However, they also exhibit an additional feature not found in ωAgaIVA-treated WT cells: a lower current threshold for eliciting Na^+ spikes compared to wild type PCs. This increase in excitability is paralleled by an increase in membrane resistance and a decrease in dendritic size (Ovsepian and Friel 2008). These results identify two distinct mechanisms by which a Ca^{2+} channel mutation can impact membrane excitability: (1) by acutely altering voltage sensitive Ca^{2+} entry in a way that can be reproduced in WT PCs by Ca^{2+} channel blockade, and (2) by altering other cellular properties, such as membrane resistance, through changes in cell morphology during development that influence how cells respond to electrical stimulation, in a way that cannot be reproduced in WT cells by acute channel blockade.

With these observations as background, we now describe effects of the *leaner* mutation on excitatory synaptic transmission in PCs.

11.2 Effects of the *Leaner* Mutation on Excitatory Synaptic Transmission

Cerebellar Purkinje neurons receive excitatory synaptic input via two distinct pathways: the parallel fiber (PF) pathway and the climbing fiber (CF) pathway (Fig. 11.1). These pathways differ in terms of their cells of origin, the information they convey, the degree of synaptic convergence, i.e. the number of presynaptic inputs that terminate on a given PC, and the strength of postsynaptic responses elicited by single fiber stimulation. PFs are the axons of granule cells (GCs) that receive input from mossy fibers (MFs) originating in various brain stem nuclei (BN) that convey information regarding body state. Each PC receives on the order of 10^5 different PF inputs that are individually weak, in the sense that the postsynaptic depolarization that results from activation of the corresponding fiber is very small (<1 mV). PF inputs can be contrasted with input from climbing fibers, which are axons of cells in the inferior olivary nucleus that relay different types of information, although there is a lack of general agreement regarding the nature of the information

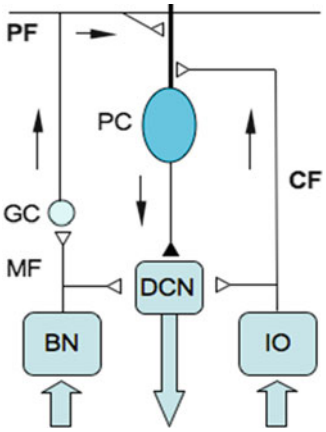

Fig. 11.1 Circuitry of the cerebellar cortex illustrating excitatory synaptic inputs received by Purkinje cells. Schematic shows the two classes of excitatory synaptic inputs received by cerebellar Purkinje cells (*PCs*). Parallel fibers (*PF*) convey information from granule cells transmitted from mossy fibers (*MF*) originating in various brain stem nuclei (*BN*). Climbing fibers (*CF*) convey information from cells in the inferior olivary nucleus (*IO*). Purkinje neurons provide inhibitory synaptic input to cells in the deep cerebellar nuclei (*DCN*) for distribution to other brain areas that participate in motor control and possibly other functions. *Arrows* indicate direction of information flow, *open and closed triangles* represent presynaptic terminals at excitatory and inhibitory synapses, respectively. For clarity, interneurons that provide inhibitory input to PCs are not shown

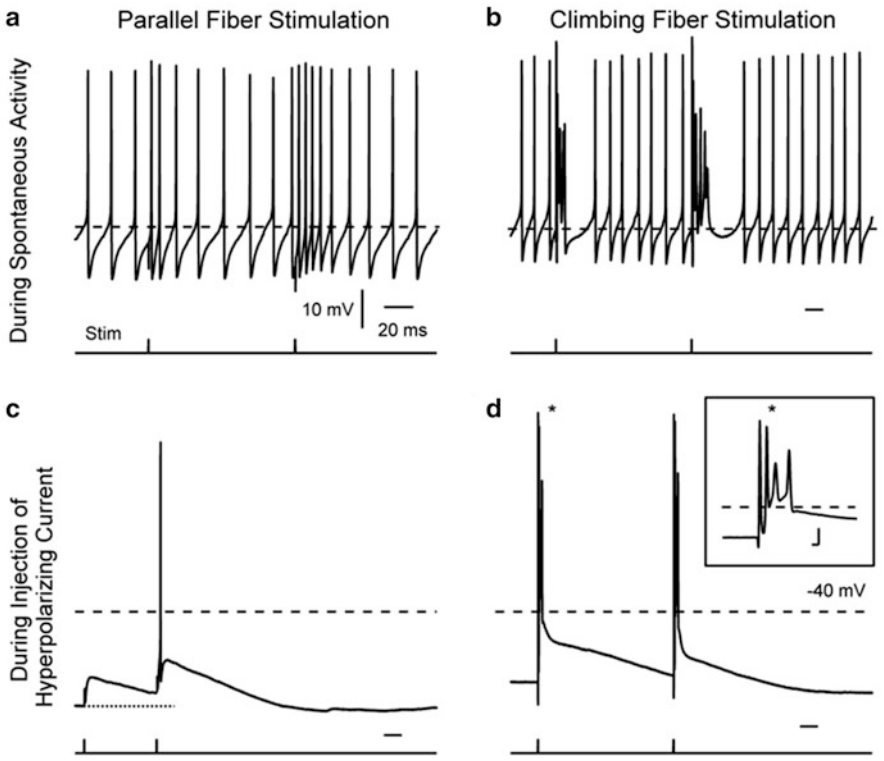

Fig. 11.2 Effects of PF and CF stimulation on tonically firing cerebellar Purkinje neurons from WT mice. *Top panels* illustrate the effects of PF (*left*) and CF (*right*) stimulation on (tonic) action potential firing in two different PCs. PF stimulation transiently increases spike frequency, while CF stimulation elicits a complex spike followed by a pause in firing. *Bottom panels* show the effects of PF and CF stimulation while injecting steady hyperpolarizing current to suppress spontaneous activity, making it possible to observe PF- and CF-EPSPs in isolation. In each panel, *top trace* shows membrane potential and *bottom trace* indicates stimulus timing (arbitrary ordinate scale). Voltage bar is the same for all panels: 10 mV. *Time bars*: 20 ms. *Dashed line*: −40 mV. Inset in panel **d** shows first complex spike on expanded scale. *Abscissa bar*: 1 ms, *ordinate bar*: 10 mV. All measurements are from P17 –21 mice. S. Liu, D. Friel unpublished results

they convey (Manto et al. 2012). Adult PCs receive input from only one CF and the postsynaptic effect of CF stimulation is strong, consisting of multiple depolarizing events that together comprise what is termed the 'complex spike'.

11.2.1 Impact of Excitatory Synaptic Stimulation on Purkinje Cells

Figure 11.2 illustrates effects of PF and CF stimulation on PCs in acute cerebellar slices prepared from WT C57BL/6J mice. To trigger excitatory synaptic

transmission at PF-PC and CF-PC synapses, extracellular stimulating electrodes were positioned near the respective input fibers and field stimuli delivered to activate PFs or CFs. Postsynaptic responses in PCs were monitored with a voltage-sensing electrode using the whole-cell current clamp technique. The top panels show how stimulation of PFs and CFs affect ongoing electrical activity in spontaneously firing PCs. At the stimulus intensity used, PF stimulation (left) produces a transient increase in spike frequency. In contrast, CF stimulation (right) triggers a complex spike followed by a 30–40 ms pause in spike activity. The bottom panel shows excitatory postsynaptic potentials (EPSPs) elicited by PF and CF stimulation in the absence of spontaneous activity. For these measurements, steady hyperpolarizing current was injected via the voltage sensing electrode to suppress spontaneous firing, thereby making it possible to observe evoked synaptic potentials in isolation; for reference, the horizontal dashed line indicates −40 mV. PF stimulation (Fig. 11.2c) produces a transient depolarization, which if sufficiently large, triggers a PC action potential. Action potentials can be elicited either with a single suprathreshold stimulus, or with a second stimulus that, by itself, is of subthreshold intensity but is suprathreshold when delivered during the declining phase of a previous EPSP, as in Fig. 11.2c. Figure 11.2d shows complex spikes elicited by climbing fiber stimulation. These electrical events consist of several fast spikes (the inset shows the first complex spike in panel D on an expanded time scale) followed by a slowly declining after-depolarization. It should be mentioned that responses to excitatory synaptic input depend on voltage, so the responses illustrated in Fig. 11.2c, d only provide an indirect indication of the electrical events initiated by stimulation while PCs fire spontaneously as in Fig. 11.2a, b.

It should be noted that Fig. 11.2a, b illustrates effects of excitatory synaptic stimulation on one of two basic modes of spontaneous activity that have been described in PCs in acute cerebellar slices: (1) tonic firing (as in Fig. 11.2), and (2) a more complex mode of activity consisting of periods of firing separated by periods of quiescence (not shown) (Womack and Khodakhah 2002). Since the focus of this review is on excitatory synaptic transmission and its sensitivity to P/Q channel mutations, the different modes of spontaneous firing in PCs will not be discussed. However, these modes of activity may be functionally important and be sensitive to P/Q channel activity. Clearly, it will ultimately be important to consider both pre-and postsynaptic effects of P/Q channel mutations when evaluating the overall effects of these mutations on cerebellar information processing and motor control.

11.2.2 Effects of the Leaner *P/Q Channel Mutation on Excitatory Synaptic Currents in Purkinje Neurons Under Voltage Clamp*

One of the most direct ways to assess functional effects of a Ca^{2+} channel mutation on synaptic transmission is to measure the postsynaptic current elicited by presynaptic stimulation while the postsynaptic cell is maintained at a fixed

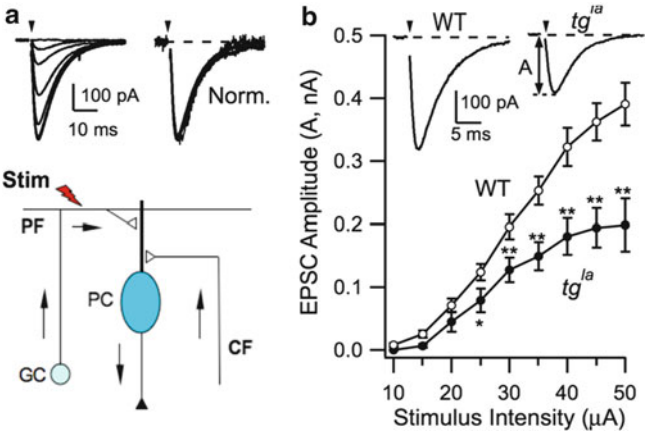

Fig. 11.3 Comparison between excitatory postsynaptic currents elicited by PF stimulation in WT and *leaner* PCs under voltage clamp. (**a**) *Top*, *Left*: PF-EPSCs from a representative WT PC elicited by an extracellular stimulating electrode positioned in the molecular layer at increasing stimulus intensities (10, 20, 25, 30, 35, and 40 μA) and at 40 μA after exposure to NBQX (10 μM) + APV (100 μM), which completely blocked transmission (*top trace*). (*Right*) After normalizing to the same amplitude, these EPSCs have indistinguishable kinetics. (**b**) Comparison between PF-EPSCs in WT and *leaner* mice. *Top*: Representative synaptic currents elicited in WT and *leaner* PCs; stimulus intensity: 40 μA. *Bottom*: Input/output curves relating PF-EPSP amplitude and stimulus intensity. *, ** indicate significant differences: $P < 0.05$, $P < 0.01$, respectively. *Arrowheads* in panels (**a**) and (**b**) indicate stimulus timing. Stimulus artifacts have been removed for clarity. Holding potential: −70 mV. Stimulus duration: 200 μs. Bicuculline (20 μM) or SR 95531 (10 μM) was included in the perfusion solution to block fast inhibitory synaptic transmission. Schematic in Panel (**a**) (*bottom*) illustrates site of stimulation (Adapted from Liu and Friel 2008)

membrane potential, e.g. under voltage clamp. Figure 11.3a illustrates excitatory postsynaptic currents (EPSCs) elicited by PF stimulation (PF-EPSCs) in a WT PC. PF-EPSCs are carried by glutamate-sensitive channels since they are completely blocked by a combination of the selective glutamate receptor antagonists NBQX and APV (top trace, Fig. 11.3a, left). Stimuli of increasing intensity activate increasing numbers of PFs, each of which makes a small contribution to the postsynaptic current, accounting for the graded relationship between stimulus intensity and PF-EPSC amplitude. It was found that PF-EPSCs in *leaner* PCs are considerably smaller than those observed in age-matched WT cells over the range of stimulus intensities examined (Fig. 11.3b); no detectable difference in time to peak or recovery time constant was detected (not shown).

Recordings of excitatory postsynaptic currents elicited by CF stimulation are shown in Fig. 11.4. Panel A compares representative CF-EPSCs elicited in PCs from WT and *leaner* mice while Panel B shows collected results, including single cell input-output curves illustrating the all-or-none nature of CF-EPSCs in both strains of PCs (left), and amplitude histograms from WT and *leaner* PCs (right). The main finding is that in contrast to PF-EPSCs, CF-EPSCs are considerably larger in *leaner* mice than they are in WT animals.

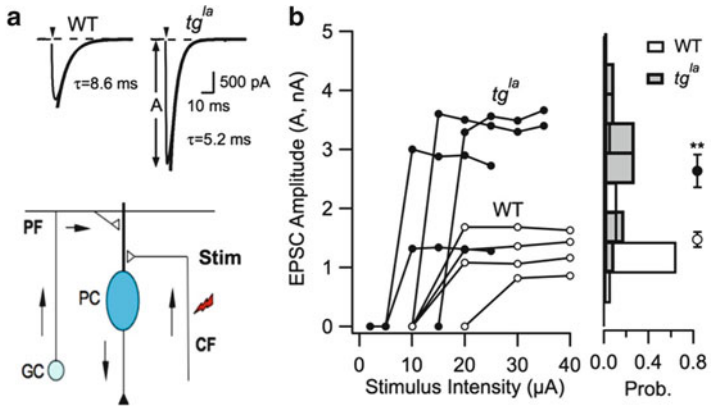

Fig. 11.4 Comparison between excitatory postsynaptic currents elicited by CF stimulation in WT and *leaner* PCs under voltage clamp. (**a**) *Top*: CF-EPSCs from representative WT (*left*) and *leaner* (*right*) PCs elicited by an extracellular stimulating electrode, in each case positioned to stimulate an all or none synaptic current. *Arrowheads* indicate stimulus timing. (**b**) *Left*: Input/output curves for individual WT and *leaner* PCs relating stimulus intensity to the amplitude of CF-EPSPs. *Right*: Distribution of CF-EPSC amplitudes in *leaner* and WT PCs. Mean amplitudes are significantly different (** $P < 0.01$). Holding potential: −70 mV. To help maintain voltage control during measurement of CF-EPSCs, the AMPA/kainate receptor antagonist NBQX was included in the extracellular solution at a subsaturating concentration (0.7 μM) to reduce EPSC amplitude, and QX-314 was included in the internal solution to block fast voltage-gated Na^+ channels. Schematic in Panel (**a**) (*bottom*) illustrates site of stimulation (Adapted from Liu and Friel 2008)

11.2.3 Are the Effects of the Leaner *Mutation on PF- and CF-EPSCs Due to Changes in Presynaptic Function?*

The observation that the *leaner* mutation modifies excitatory postsynaptic currents at PF-PC and CF-PC synapses raises questions regarding the sites at which the mutation might exerts its effects on transmission. Previous work has shown that in WT mice, Ca^{2+} entry through presynaptic P/Q channels contributes to release of excitatory neurotransmitter at PF-PC (Mintz et al. 1995; Matsushita et al. 2002), and to a lesser extent at CF-PC synapses (Regehr and Mintz 1994; Matsushita et al. 2002), raising the possibility that altered presynaptic Ca^{2+} entry plays a role in modifying synaptic currents in *leaner* mice. One approach to assessing a presynaptic locus of action is to investigate responses to paired synaptic stimuli. When two such stimuli are separated by a short time interval (e.g. on the order of a second), the response to the second stimulus may be influenced by the first stimulus due to presynaptic effects set into motion by the first stimulus. Altered synaptic transmission in the aftermath of a given stimulus resulting from presynaptic changes induced by that stimulus is called short-term synaptic plasticity. A convenient measure of short-term plasticity is the paired-pulse ratio, the ratio of the amplitudes

of the second to the first EPSC evoked by a pair of stimuli with a given interstimulus interval. If the paired-pulse ratio is greater than one, synaptic facilitation has occurred, while if it is less than one, synaptic depression is indicated.

Synaptic facilitation is thought to result from the buildup of presynaptic Ca^{2+} levels during repetitive stimulation (Zucker and Regehr 2002; Mochida et al. 2008). For example, if a second stimulus occurs before the presynaptic Ca^{2+} concentration ($[Ca^{2+}]_i$) has recovered following the first stimulus, the absolute $[Ca^{2+}]_i$ level reached in response to the second stimulus may be higher than that achieved by the first stimulus, owing to additive effects of stimulus-induced Ca^{2+} entry. All other things being equal (such as the number of releasable synaptic vesicles, Ca^{2+} sensitivity of vesicular release, and postsynaptic responsiveness to transmitter), a larger increase in presynaptic $[Ca^{2+}]_i$ would be expected to trigger more neurotransmitter release and a larger postsynaptic response, compared to the first stimulus, an effect that is amplified by the steep $[Ca^{2+}]_i$ dependence of exocytosis (Dodge and Rahamimoff 1967). Residual elevations in presynaptic $[Ca^{2+}]_i$ could potentially have additional effects contributing to facilitation, e.g. modulation of Ca^{2+} channels leading to enhanced Ca^{2+} entry in response to the second stimulus, inhibition of Ca^{2+} removal or sequestration, saturation of Ca^{2+} buffers, enhanced Ca^{2+} release from intracellular stores, or even changes in the Ca^{2+} sensitivity of the exocytotic process.

A major cause of short-term depression is depletion of releasable synaptic vesicles (Zucker and Regehr 2002). If a second stimulus occurs before the pool of readily releasable vesicles is fully replenished after the first stimulus, fewer vesicles will be available for release, with the consequence that, all other things being equal (such as the presynaptic Ca^{2+} concentration, Ca^{2+} sensitivity of vesicular release, and postsynaptic responsiveness to transmitter), a second stimulus will trigger release of fewer synaptic vesicles, resulting in a smaller postsynaptic response. Other presynaptic mechanisms that could potentially contribute to depression include modulation of Ca^{2+} channels leading to reduced Ca^{2+} entry in response to the second stimulus, Ca^{2+} dependent slowing of vesicle replenishment, and reduced Ca^{2+} sensitivity of exocytosis. For a given synapse, the relative contributions from facilitation and depression determine the overall characteristics of short-term plasticity, quantified by the paired-pulse ratio (Dittman et al. 2000; Zucker and Regehr 2002).

11.2.4 Assessment of Short-Term Plasticity at PF-PC Synapses

Previous studies have shown that short-term synaptic plasticity at PF-PC synapses in WT mice is dominated by facilitation (Konnerth et al. 1990), possibly because vesicle release probability is small enough that the effects of residual Ca^{2+} outweigh the effects of vesicle depletion. Figure 11.5 compares paired-pulse facilitation at PF-PC synapses in WT and *leaner* PCs. It was found that mutant PCs display larger paired-pulse ratios than WT neurons, indicating enhanced facilitation.

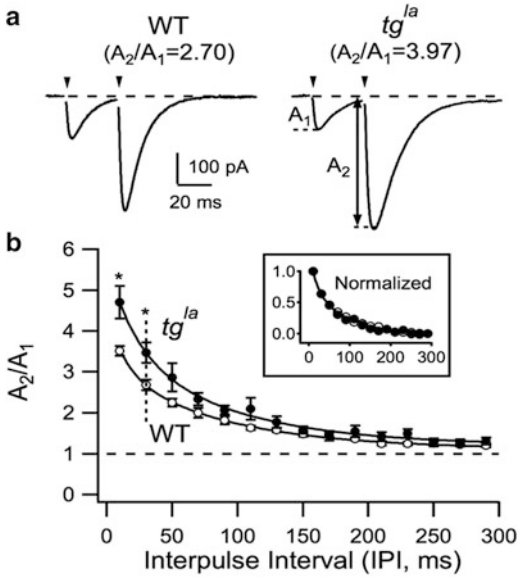

Fig. 11.5 Paired-pulse facilitation is enhanced at PF-PC synapses in *leaner* mice. (**a**) Comparison between PF-EPSCs elicited by two 35 μA stimuli separated by 30 ms in representative WT (*left*) and *leaner* (*right*) PCs. This pathway displayed enhanced paired-pulse facilitation (PPF) in *leaner* mice, quantified as the ratio of the amplitudes of the second to the first EPSC (A_2/A_1). (**b**) Time course of recovery from PPF. Recoveries could be described by biexponential functions with limiting values of unity (see *smooth curves*). *Dashed vertical line* indicates the interpulse interval (30 ms) separating stimuli in panel **a**. Inset compares recovery kinetics after normalizing A_2/A_1 measurements to the values at the shortest interpulse interval (20 ms) and subtracting unity. Holding potential: −70 mV. * $P < 0.025$ (Adapted from Liu and Friel 2008)

The most parsimonious explanation of this finding is that PF-EPSCs are smaller in *leaner* mice because presynaptic Ca^{2+} entry is reduced, leading to less neurotransmitter release over the population of PFs contributing to the overall postsynaptic response. With transmission occurring at fewer synapses in response to the first stimulus, more presynaptic boutons would have a full complement of readily releasable vesicles at the time of the second stimulus, which would tend to increase the paired-pulse ratio. The observation that the kinetics of recovery after paired-pulse facilitation is indistinguishable in *leaner* and WT cells indicates that the processes underlying recovery from facilitation, such as presynaptic Ca^{2+} diffusion away from release sites, extrusion and sequestration, are not strongly affected by the *leaner* mutation.

While these results are consistent with the concept that reduced presynaptic Ca^{2+} entry plays a role in attenuating synaptic strength at PF-PC synapses, it is important to consider other potential contributing factors. For example, Herrup and Wilczynski (Herrup and Wilczynski 1982) found that from roughly postnatal day 10 there are fewer granule cells in *leaner* cerebella compared to WT mice. Since PFs are axons

of granule cells, a reduction in the number of GCs would be expected to reduce the number of PFs contributing to PF-EPSCs, thereby attenuating these synaptic currents. Another possibility is that the *leaner* mutation reduces the sensitivity of PFs to field stimuli like those used to elicit PF-EPCS, such that for a given stimulus intensity, fewer PFs contribute to PF-EPSCs in *leaner* mice. While this possibility has not been rigorously excluded, it is inconsistent with the observation that in *leaner* mice the input/output curve seems to level off at a lower level than in WT mice (see Fig. 11.3b). A third possibility is that the mutation reduces postsynaptic sensitivity to glutamate (Kodama et al. 2006), with the consequence that at each synapse, postsynaptic currents would be smaller in *leaner* compared to WT mice, a possibility that has yet to be assessed unequivocally. It should be noted, however, that while each of the three mechanisms listed above could contribute to the reduction in synaptic strength at PF-PC synapses, none of them account for the observed increase in paired-pulse facilitation.

11.2.5 Assessment of Short-Term Plasticity at CF-PC Synapses

As mentioned above, Ca^{2+} entry through P/Q channels contributes to neurotransmitter release at CF-PC synapses. It is therefore puzzling that the *leaner* mutation leads to an *increase* in CF-EPSCs, since reduced Ca^{2+} entry through mutant channels would be expected to have the opposite effect, as observed at PF-PC synapses. To address whether the increase in CF-EPSC size is associated with enhanced synaptic release, we again turned to analysis of short-term plasticity. As reported in previous studies, transmission at CF-PC synapses shows paired-pulse *depression* (Konnerth et al. 1990). This is thought to reflect the high probability of vesicular release at CF synapses such that vesicle depletion dominates short-term plasticity (Xu-Friedman and Regehr 2004). If the effect of the *leaner* mutation on CF-EPSCs is due to an increase in the amount of neurotransmitter that is released at individual CF-PC synapses, it would be expected to reduce the number of vesicles that are available for release following a first stimulus, thereby exaggerating depression, which would reduce the paired-pulse ratio.

Figure 11.6a (left) shows CF-EPSCs in WT mice elicited by a pair of stimuli separated by 80 ms. Synaptic currents elicited in a representative *leaner* PC using the same pulse protocol is shown in Fig. 11.6a (right). Despite the larger synaptic current amplitudes seen in mutant mice, the paired-pulse ratio is indistinguishable in the two mouse strains. This is true for all interpulse intervals tested (Fig. 11.6b). This finding supports the conclusion that the increase in CF-EPSC size found in *leaner* mice is not due to enhanced neurotransmitter release at single CF-PC synapses.

One factor that could contribute to the larger CF-EPSCs seen in *leaner* mice is an increase in the number of CFs that innervate individual PCs, which has been reported to result from other disruptions of ion channel function (Miyazaki et al. 2004; Kodama et al. 2006). This could increase the size of the EPSC elicited by a maximally effective stimulus. However, such a change would be expected to

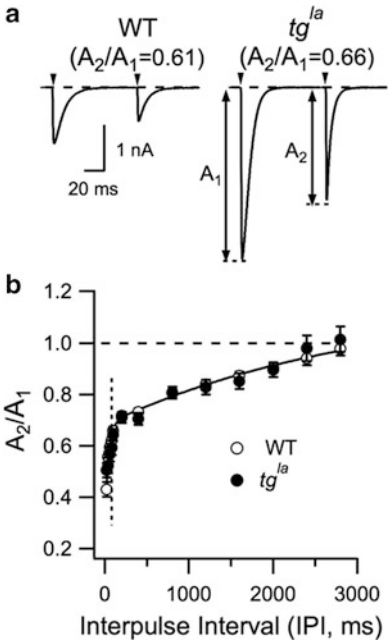

Fig. 11.6 Paired-pulse depression at CF-PC synapses is unchanged in *leaner* mice. (**a**) Comparison between EPSCs elicited by two 30 μA stimuli separated by 80 ms in representative wild type (*WT*, *left*) and *leaner* (*right*) PCs. Both WT and *leaner* PCs showed paired-pulse depression (PPD) at CF synapses, quantified as the ratio of the amplitudes of the second to the first EPSCs (A_2/A_1). (**b**) Time course of recovery from PPD. Recoveries follow biexponential time courses with limiting values of unity (see *smooth curves*) that are indistinguishable in the two cell populations. *Dashed vertical line* indicates the interpulse interval (80 ms) separating stimuli in (**a**). Results in (**b**) are based on analysis of 15 PCs in 8 WT mice (except for the 2,800 ms time point where n = 8) and 8–11 PCs in 4 *leaner* mice. Holding potential: −70 mV (Adapted from Liu and Friel 2008)

transform the input-output relation from a single step to a multi-step function. In contrast to this expectation, the majority of CF-EPSCs in *leaner* mice were triggered in an all-or-none manner (Fig. 11.4b). While fluctuations in CF-EPSCs potentially reflecting innervation of PCs by multiple CFs were observed in 36 % of *leaner* PCs compared to 6 % of WT cells, in each of these cases the largest of the observable CF-EPSC steps in *leaner* still exceeded the mean WT CF-EPSC amplitude. This indicates that while it is possible that the *leaner* mutation increases the number of CFs terminating on individual PCs, such an increase cannot by itself explain the larger CF-EPSC amplitudes observed in *leaner* PCs.

What could account for the observed enhancement of synaptic strength without changes in short term plasticity? Possibilities include an increase in the number of synapses between individual CFs and their postsynaptic PC without changes in presynaptic function, an increase in postsynaptic sensitivity at CF-PC synapses, and/or an increase in the number of transmitter molecules per synaptic vesicle.

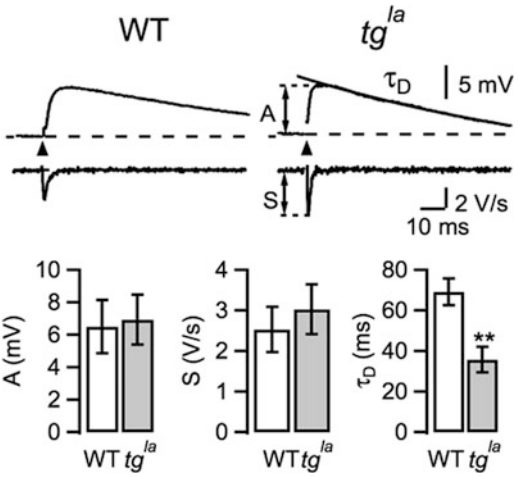

Fig. 11.7 *leaner* PCs respond to PF stimulation with subthreshold membrane potential responses resembling those found in WT cells (A) *Top*: Comparison between EPSPs elicited by PF stimulation (40 μA) in WT (*left*) and *leaner* (tg^{la}) PCs at hyperpolarized voltages. *Upper traces*: membrane potential (*V*); *lower traces*: −dV/dt. *Bottom* (*left to right*): Mean EPSC amplitude (A), maximal slope (S) and decay time constant (τ_D) from 4 WT and 4 *leaner* PCs (obtained from 3 WT and 2 mutant mice, respectively); responses elicited by stimuli within the range 40–50 μA were pooled. (** $P < 0.01$). Tick mark to left of voltage traces: −65 mV (Adapted from Liu and Friel 2008)

11.2.6 Effects of the Leaner *Mutation on Excitatory Synaptic Potentials in Purkinje Neurons Under Current Clamp*

Having described effects of the *leaner* mutation on postsynaptic currents elicited by PF and CF stimulation under voltage clamp, it is important to consider potential functional consequences of these changes. Arguably one of the most important functional roles of excitatory synaptic transmission is to depolarize the membrane potential of the postsynaptic cell. How the membrane potential changes in response to synaptic transmission depends on the postsynaptic conductance that is activated, the associated current, and the intrinsic electrical properties of the postsynaptic cell at the time of transmission, which depend on the channels that are present and the membrane capacitance. Previous work indicates that the *leaner* mutation affects intrinsic electrical properties of PCs (Ovsepian and Friel 2008), suggesting that the effects of the mutation on synaptic potentials might reflect the combination of altered synaptic currents and altered intrinsic properties.

Figure 11.7 compares excitatory postsynaptic potentials (EPSPs) elicited by stimulation of the PF pathway in WT and *leaner* PCs at the same intensity. Despite the observed ∼50 % reduction in PF-EPSC amplitude in *leaner* PCs, EPSPs

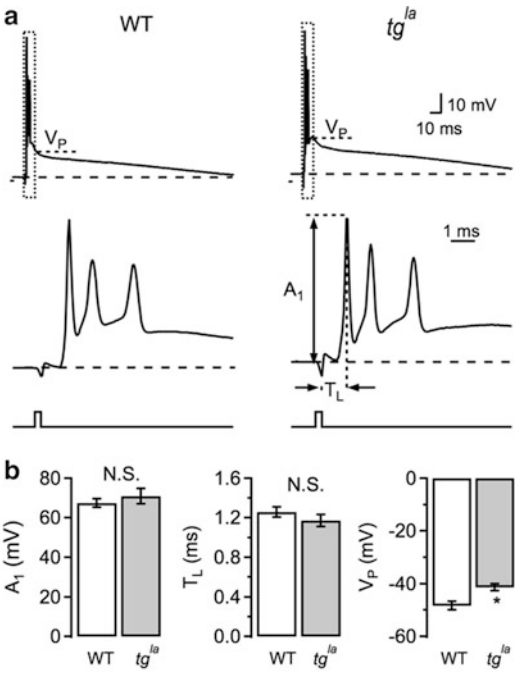

Fig. 11.8 *leaner* PCs respond to CF stimulation with complex spikes closely resembling those found in WT cells (**a**) *Top*: Representative CF-stimulation evoked complex spikes in PCs in WT (*left*) and *leaner* (*right*) PCs at hyperpolarized membrane potentials. Plateau level (V_p) is indicated by *horizontal dashed line*. Bottom: Complex spikes from top panels (see *regions enclosed by dotted rectangles*) on expanded time scale, along with definitions of amplitude (A_1) and latency (T_L) of the first spike. *Tick mark to left* of voltage traces: −60 mV. (**b**) Collected results from 8 WT cells (N = 3) and five *leaner* cells (N = 3) describing mean amplitude (A_1), spike latency (T_L) and plateau level (V_p) (* $P < 0.05$) (Adapted from Liu and Friel 2008)

elicited in *leaner* and WT PCs are very similar, without detectable differences in amplitude or maximal rate of rise of the voltage. The only EPSP property that was systematically different was the smaller decay time constant in mutant PCs compared to WT cells.

Figure 11.8 presents a similar comparison between CF-EPSPs (i.e. complex spikes) in WT and *leaner* PCs. Despite the nearly twofold enhancement of CF-EPSCs in *leaner* Purkinje neurons, complex spikes elicited by CF stimulation in *leaner* and WT PCs are virtually indistinguishable, both in terms of their amplitudes and latencies. The only difference that was detected was the size of the after depolarization following the spikes, which was significantly more depolarized in *leaner* PCs than in WT cells. Overall, in view of the effects of the *leaner* mutation on EPSCs, the most conspicuous feature of the effect of the mutation on excitatory synaptic transmission is how small it is.

11.3 Discussion

This review addresses a question with broad relevance for studies of genetic disease: What is the impact of a mutation in a gene that is expressed in multiple cellular contexts? The focus in this review is the *Cacna1a* gene encoding the pore-forming subunit of P/Q type Ca^{2+} channels. These channels are expressed throughout the nervous system in multiple cell populations where they regulate a variety of processes, including neurotransmitter release and membrane excitation, with diverse consequences for nervous system function. Here we focused on the impact of the *leaner* mutation on excitatory synaptic transmission, specifically via the two pathways that provide excitatory input to Purkinje neurons. These pathways are (a) differentially affected at the level of postsynaptic currents measured under voltage clamp, and yet are (b) virtually insensitive to the mutation at the level of postsynaptic potentials measured under current clamp conditions.

Given previous work describing effects of the *leaner* mutation on intrinsic membrane properties of PCs, it is worth considering whether the observed changes in synaptic currents, when taken together with the changes in intrinsic properties, reconcile findings (a) and (b) above. The *leaner* mutation leads to an increase in membrane resistance in PCs. This increase is paralleled by a reduction in dendritic size, providing a potential structural basis for the increase in resistance. Such a change would be expected to increase the size of postsynaptic potentials elicited by excitatory synaptic currents, thereby tending to normalize EPSP amplitudes in the face of reduced EPSC amplitude. Additionally, a reduction in dendritic size might be expected to shift the location of PF-PC synapses so they are, on average, closer to the cell body, resulting in less dendritic signal attenuation. Both factors may contribute to the observed increase in gain relating synaptic potentials to synaptic currents and help reconcile observations (a) and (b) above.

The *leaner* mutation has also been found to impair the ability of PCs to generate Ca^{2+} spikes, even in response to strong stimulation (Ovsepian and Friel 2008), presumably because the density of dendritic P/Q Ca^{2+} current is insufficient for the regenerative Ca^{2+} entry required for dendritic Ca^{2+} spikes. Since the depolarizing phase of dendritic Ca^{2+} spikes overlap in time with somatic complex spikes (Davie et al. 2008), it is possible that Ca^{2+} entry through P/Q type channels normally provides an important component of the inward current linking CF stimulation to somatic complex spike generation. In this case, reduced Ca^{2+} entry through P/Q type Ca^{2+} channels in *leaner* PC dendrites would diminish the effectiveness of CF stimulation in generating somatic complex spikes. In this case, enhancement of CF-EPSCs would compensate for reduced Ca^{2+} entry, thereby preserving CF stimulus-induced complex spikes.

Given that the *leaner* mutation only weakly affects excitatory synaptic potentials in PCs, what accounts for the effect of the mutation on cerebellar motor control? While the answer is not yet available, it is likely to involve multiple effects, including changes in intrinsic membrane properties of PCs (Ovsepian and Friel 2008) that affect spontaneous action potential generation (Walter et al. 2006),

changes in inhibitory synaptic control of PCs (Ovsepian and Friel 2012), and modifications in the number and intrinsic properties of other neurons that comprise circuits that participate in cerebellar motor control.

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Chapter 12
$Ca_V2.1$ (P/Q) Voltage Activated Ca^{2+} Channels and Synaptic Transmission in Genetic and Autoimmune Diseases

Osvaldo D. Uchitel

Abstract $Ca_V2.1$ (P/Q type) Ca^{2+} channels have a fundamental role mediating fast transmitter release at central and peripheral synaptic terminals. Various neurological diseases have been attributed to genetic and autoimmune malfunctioning of P/Q channels, including ataxia, migraine and myasthenic syndromes. This chapter focuses on recent advances on the understanding of the pathogenic mechanisms underlying these disorders.

Keywords $Ca_V2.1$ subunits • P/Q type current • Channelopathy • Ataxia • Migraine • Myasthenic syndromes

12.1 Introduction

Voltage-gated Ca^{2+} channels (VGCCs) transduce electrical signals into local intracellular Ca^{2+}transients thus regulating intracellular processes such as enzyme activation, gene expression, neurite outgrowth or retraction and neurotransmission (Catterall 2011). It is well established that a high-power association relates neurotransmitter release probability to the concentration of presynaptic Ca^{2+}. Activated by an action potential (AP), VGCCs can mediate Ca^{2+} entry into presynaptic terminals. Once inside the terminal, Ca^{2+} ions rapidly bind to endogenous intracellular buffers and could trigger Ca^{2+} discharge from internal Ca^{2+} stores. The resulting space-time profiles of free Ca^{2+} determines the time-course and probability of neurotransmitter release through the interaction with molecular release triggers strategically located nearby release sites. Following a rapid Ca^{2+} concentration transient, excess Ca^{2+} has to be removed from the cytosol through a process

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involving Ca^{2+} uptake by the endoplasmic reticulum stores, sequestration by mitochondria, and/or extrusion into the extracellular medium.

In this chapter we present an overview on how transmitter release is affected when VGCCs, one of the major players involved in Ca^{2+}-dependent presynaptic regulation of neurotransmitter, are affected by genetic or autoimmune mechanisms related to human diseases. In particular, we focus on the expanding phenotypic spectrum of diseases associated with dysfunction of the $Ca_V2.1$ Ca^{2+} channel, and examine the pathophysiological basis of these disorders.

12.2 Voltage Gated Ca^{2+} Channels (VGCCs) Subunit Organization and Classification

VGCCs are composed by a $\alpha_1$1–10 subunit forming the Ca^{2+} selective channel, and several accessory subunits, $\alpha_2\delta$, β_{1-4}, and γ, with anchorage and regulatory functions. Based on their unique electrophysiological and pharmacological properties, and the type of α_1 subunit, VGCCs are divided into five classes: $Ca_V1.1$–$Ca_V1.4$ (L-type), $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type), $Ca_V2.3$ (R-type) and $Ca_V3.1$–3.3 (T-type) Ca^{2+} channels (Catterall 2011).

The VGCC $\alpha_2\delta$ and β subunits are traditionally considered to be auxiliary subunits that enhance channel trafficking, increase the expression of functional Ca^{2+} channels at the plasma membrane and influence the channel biophysical properties. Accumulating evidence indicates that these subunits may also have roles in the nervous system that are not directly linked to the Ca^{2+} channel function. For example, β subunits may act as transcriptional regulators, and certain $\alpha_2\delta$ subunits may have a function in synaptogenesis (Dolphin 2009, 2012; Perez-Reyes 2010).

$Ca_V2.1$ channels are located throughout the mammalian brain and spinal cord at presynaptic terminals and at somatodendritic membranes (Mintz et al. 1992; Westenbroek et al. 1995, 1998; Catterall 1998). In vertebrates, the CACNA1A gene encoding $Ca_V2.1$ channels undergoes alternative splicing at multiple loci in an age-, gender-, and species-dependent manner (Bourinet et al. 1999; Soong et al. 2002; Chaudhuri et al. 2005; Chang et al. 2007). This mechanism results in multiple $Ca_V2.1$ splice variants with different outcomes in neuronal distribution and subcellular localization, biophysical properties, and sensitivity to the specific blocker ω-agatoxin-IVA. In addition, functional diversity of $Ca_V2.1$ channels is generated by the combination of $Ca_V2.1$ $\alpha1$ subunits with various auxiliary β and $\alpha2\delta$ subunits (Luvisetto et al. 2004). Indeed, there are many experimental evidences suggesting a large functional and pharmacological variability of native $Ca_V2.1$ channels (Randall and Tsien 1995; Tottene et al. 1996; Mermelstein et al. 1999; Dolphin 2009).

Among the presynaptic Ca^{2+} channels, $Ca_V2.1$ channels are also unique for their capacity to interact and be modulated in a complex manner by a number of intracellular Ca^{2+}-binding proteins (Catterall and Few 2008). As a result, $Ca_V2.1$

channels may exhibit either Ca^{2+}-dependent inactivation (CDI) or Ca^{2+}-dependent facilitation (CDF), depending on the current physiological condition. Moreover, Ca^{2+}-dependent regulation of presynaptic $Ca_V2.1$ channels may play a crucial role in short-term synaptic plasticity during trains of action potentials (Cuttle et al. 1998; Inchauspe et al. 2004; Mochida et al. 2008; Muller et al. 2008).

12.3 Presynaptic VGCCs Associated with Transmitter Release

Presynaptic VGCCs are part of a sophisticated regulation system of presynaptic pathways that influence the kinetic and mechanism of neurotransmitter release. VGCCs, SNARES and a variety of proteins associated with synaptic vesicles function as a building block system with a highly conserved basic mechanism and variable single modules that ultimately determine the properties of the synapse. Such a system explains the great variability of neurotransmitter release among different neurons, where presynaptic VGCCs represent an important part of the individual variable modules.

P/Q-type, N-type and, to some extent, R-type channels are highly expressed at presynaptic nerve terminals where their activities evoke neurotransmitter release. They play a prominent role in initiating action-potential-evoked neurotransmitter release both at peripheral neuromuscular junctions and central synapses, mainly within the cerebellum, brainstem, and cerebral cortex (Takahashi and Momiyama 1993; Wheeler et al. 1994; Katz et al. 1996; Iwasaki et al. 2000; Nudler et al. 2003; Trimmer and Rhodes 2004; Kamp et al. 2012). Even when pharmacological and electrophysiological studies in rodent brain slices have revealed that P/Q-, N- and R-type channels cooperate in controlling release at many central excitatory synapses, P/Q-type channels have a dominant role, partly because of their more efficient coupling to the exocytotic machinery (Mintz et al. 1995; Qian and Noebels 2000. 2001; Inchauspe et al. 2004; Li et al. 2007).

A relationship between the type of neurotransmitter released and the type of VGCCs evoking exocytosis might exist. In the majority of GABA-releasing inhibitory neurons, GABA release is mediated by Ca^{2+} influx through P/Q-type VGCCs (Poncer et al. 1997; Iwasaki et al. 2000; Brager et al. 2003; Hefft and Jonas 2005; Tecuapetla et al. 2005; Zaitsev et al. 2007; Lonchamp et al. 2009). In contrast, glutamate release is often jointly mediated by both P/Q- and N-type VGCCs in the vast majority of glutamatergic cortical and cerebellar synapses (Regehr and Mintz 1994; Turner et al. 1995; Vazquez and Sanchez-Prieto 1997; Iwasaki et al. 2000; Rozov et al. 2001; Millan and Sanchez-Prieto 2002; Ladera et al. 2009) with a few exceptions (Ali and Nelson 2006).

At the neuromuscular junction (and at many central synapses), there is a developmental change in the VGCCs subtypes mediating synaptic transmission,

whereby the relative contribution of $Ca_V2.1$ channels increases with early postnatal age, until it becomes exclusively dependent on P/Q-type channels (Rosato Siri and Uchitel 1999; Iwasaki et al. 2000; Rosato-Siri et al. 2002; Urbano et al. 2002). P/Q-type channels also mediate about 40 % of the AP-evoked Ca^{2+} influx in dendritic spines and shafts of layer 2/3 cortical PC (Koester and Sakmann 2000) and contribute to the regulation of the intrinsic firing of the same neurons through activation of different Ca^{2+}-dependent K^+ (K_{Ca}) channels (Pineda et al. 1998).

12.4 Human Genetic Disorders Related to $Ca_V2.1$ (P/Q) Ca^{2+} Channels

At least three conditions, episodic ataxia type 2 (EA2), familial hemiplegic migraine type 1 (FHM1) and spinocerebellar ataxia type 6 (SCA6) have been described in association with mutations in the CACNA1A gene encoding Cav2.1 Ca^{2+} channels. Although each disorder is distinctive, they exhibit considerable overlap in clinical features, both between individuals and within affected families. For example, many patients with familial hemiplegic migraine show permanent cerebellar signs (Wessman et al. 2007), whereas more than half of the patients with EA2 meet the International Headache Society criteria for the diagnosis of migraine and some other patients with EA2 experience episodes of paresis (Jen et al. 2004). EA2 and FHM1 disorders are thought to be the result of impaired regulation of neuronal excitability, but their underlying mechanisms are unknown. Deciphering these mechanisms could provide important insights into the physiological role of the Ca_V channels, and the neural changes mediating the occurrence of paroxysmal attacks (i.e., short, frequent and stereotyped symptoms that can be observed in various clinical conditions).

More than a decade ago, the disease locus of EA2 was mapped to chromosome 19p (Kramer et al. 1995; Vahedi et al. 1995) in the same region as the disease locus for FHM1 (Joutel et al. 1993). A Ca^{2+} channel gene CACNA1A was mapped to this locus on chromosome 19p. Ophoff and colleagues characterized the genomic structure of CACNA1A and identified missense mutations in FHM1 and truncation (frame shift and splice site) mutations in EA2 (Ophoff et al. 1996). Glutamine-encoding CAG-repeat expansion in CACNA1A causes spinocerebellar ataxia type 6 (SCA6), a dominantly inherited pure cerebellar ataxia syndrome of late onset (Zhuchenko et al. 1997).

12.4.1 Migraine and the Familial Hemiplegic Migraine

Migraine is a common, chronic neurovascular disorder, typically characterized by recurrent attacks of disabling headaches and associated autonomic symptoms.

Twelve percent of the general population has on average one to two migraine attacks per month and treatments are frequently unsatisfactory. The etiology of migraine is multifactorial (for reviews, see Goadsby 2002; Pietrobon and Striessnig 2003). Migraine pain is likely to be caused by activation of the trigeminovascular system, which primarily consists of trigeminal afferents innervating meningeal blood vessels, the trigeminal nerve, and brainstem nuclei that modulate sensory signal transmission.

Up to one-third of the patients perceive an "aura" prior to migraine headaches, defined as a transient visual, sensory, language, or motor disturbance which signals that the headache will soon follow. Neuroimaging findings indicate that migraine aura is due to the existence of a cortical spreading depression (CSD) involving a wave of sustained strong neuronal depolarization that slowly progresses across the cortex, generating a transient intense spike activity followed by long-lasting neural suppression (Lauritzen 1994; Cutrer et al. 1998; Bowyer et al. 2001; Hadjikhani et al. 2001; Eikermann-Haerter and Ayata 2010). In animal studies, CSD can activate the meningeal trigeminal nociceptive afferents and evoke alterations in the meninges and brainstem, consistent with the development of headaches (Bolay et al. 2002; Ayata and Moskowitz 2006; Eikermann-Haerter et al. 2011).

Migraine often runs in families (Kors et al. 2004; Haan et al. 2005), therefore genetic research in the field of migraines has focused on the identification of genes involved in familial hemiplegic migraine (FHM), a rare monogenic subtype of migraines with aura. Three genes have been identified so far: FHM-2, caused by mutations in the ATP1A2 gene (De Fusco et al. 2003) encoding the α_2-subunit of sodium-potassium pumps present in glial cells, and FHM-3, originated by mutations in the SCN1A gene (Dichgans et al. 2005) encoding the pore-forming α_1-subunit of neuronal $Na_V1.1$ voltage gated sodium channels.

Lastly, FHM-1 is caused by a spontaneous missense mutation in the CACNA1A gene encoding the ion-conducting, pore-forming α_{1A} subunit of $Ca_V2.1$ VGCCs (Ophoff et al. 1996). Over 50 CACNA1A mutations have been associated with a wide range of clinical phenotypes (Kors et al. 2004; Haan et al. 2005). These include pure forms of FHM-1 (Ophoff et al. 1996), combinations of FHM-1 with various degrees of cerebellar ataxia (Ducros et al. 2001) or fatal coma due to excessive cerebral edema (Kors et al. 2001), and disorders not associated with FHM such as episodic ataxia type 2 (Jen et al. 2004), progressive ataxia (Yue et al. 1997), spinocerebellar ataxia type 6 (Zhuchenko et al. 1997), absence (Imbrici et al. 2004) and generalized epilepsy (Jouvenceau et al. 2001; Haan et al. 2005). Apart from the characteristic transient hemiparesis, typical attacks of FHM-1 are identical to those of the common forms of migraine with aura (Thomsen et al. 2002). In addition, more than two-thirds of patients with FHM also have episodes of "normal typical migraine". Interestingly, in several FHM families, FHM-1 CACNA1A mutations also were found in family members who had only "normal" not-paretic migraine but no FHM. This suggests that gene mutations for FHM may also be responsible for the common forms of migraine, probably due to different genetic and no-genetic modulating factors. All of these characteristics make FHM-1 a promising model to study the pathogenesis of the common forms of migraine.

The functional consequences of FHM-1 mutations (including R192Q and S218L) have been investigated by expressing recombinant human $Ca_V2.1$ channel subunits in heterologous systems with controversial results since both loss-of-function and gain-of-function phenotypes have been reported (Kraus et al. 1998; Hans et al. 1999; Kraus et al. 2000; Tottene et al. 2002; Cao et al. 2004; Barrett et al. 2005; Cao and Tsien 2005). However, analysis of single-channel properties of human $Ca_V2.1$ channels carrying FHM-1 mutations revealed a consistent increase in channel open probability and in single channel VGCCs influx over a broad voltage range, mainly due to a shift of channel activation to more negative voltages (Hans et al. 1999; Tottene et al. 2002; Mullner et al. 2004; Tottene et al. 2005). Such conflicting results obtained from heterologous expression systems suggest that the analysis of Ca^{2+} channels and synaptic transmission in their native neuronal environment and at their endogenous level of expression in knock-in (KI) mouse models would most likely be a powerful tool to understand the pathogenesis of diseases like FHM. Such models will also allow the evaluation of the consequences of FHM-1 mutations on mechanisms involved in migraine, such as neurotransmission and cortical spreading depression. The generation of a KI mouse carrying the mild R192Q and the more severe clinical mutation S218L allowed the first analysis of mutant channels expressed at their endogenous level in neurons (van den Maagdenberg et al. 2004; Tottene et al. 2005; Kaja et al. 2010).

12.4.1.1 Effects of FHM-1 Mutations on Neuronal Interactions: From Whole Brain to Synaptic Terminals

Functional analysis in their normal environment revealed a pure gain-of-function effect on Ca^{2+} channel current, including a negative shift in $Ca_V2.1$ channel activation and increased synaptic transmission at the neuromuscular junction in R192Q and S218L mutants (van den Maagdenberg et al. 2004; Kaja et al. 2010). The $Ca_V2.1$ current density in cerebellar granule cells and cortical pyramidal neurons of the R192Q KI mice was larger than that in wild type neurons in a broad voltage range, and was similar to wild type at more positive voltages (van den Maagdenberg et al. 2004; Tottene et al. 2009). The changes in $Ca_V2.1$ current density measured in neurons from KI mice indicate that channels from genetically modified mice have a gain-of-function phenotype similar to that established for human channels (Hans et al. 1999; Tottene et al. 2002; Mullner et al. 2004; van den Maagdenberg et al. 2004; Tottene et al. 2005) and that the number of functional channels in the membrane is not altered by the mutation. The FHM-1 KI data suggest that an increased glutamate release from cortical excitatory synapses as a consequence of gain-of-function of $Ca_V2.1$ channels might underlie the enhanced susceptibility of the migraine brain for CSD and aura and reinforce the hypothesis that migraine is associated with neuronal hyperexcitability at the cortical and, possibly, brainstem level (Pietrobon 2005).

Using microcultures and brain slices from FHM-1 mice, Tottene et al. (2009) have shown increased probability of glutamate release at cortical layer 2/3 pyramidal cells. Intriguingly, neurotransmission from inhibitory fast-spiking interneurons appeared unaltered, despite being mediated by P/Q-type channels (i.e., carrying the FHM-1 mutation) (Tottene et al. 2009). This abnormal balance of cortical excitation-inhibition was associated with the increased susceptibility for CSD in the KI mice, but the underlying mechanism changing synaptic strength by the R192Q mutation is not yet fully understood. Interestingly, these FHM-1 gain-of-function missense mutations characteristically occlude CDF of human $Ca_V2.1$ channels in both recombinant preparations and cerebellar Purkinje cells (Adams et al. 2010), suggesting that FHM-1 gain of function missense mutations of $Ca_V2.1$ channels favors a constitutively facilitated state that prevents further Ca^{2+}- dependent calmodulin mediated channel facilitation. It is hypothesized that a disruption of this form of $Ca_V2.1$ CDF may cause the cerebellar ataxia-associated FHM-1, due to an imbalance between excitatory and inhibitory inputs to the cerebellar Purkinje cells. This disruption suppresses the intrinsic pacemaker activity of these cells, thus leading to motor deficits (Adams et al. 2010).

Recent detailed studies at the calyx of Held of the KI R192Q mouse have revealed interesting features on the modulation of the mutated Ca^{2+} currents in their natural environment (Inchauspe et al. 2010). The calyx of Held is a giant glutamatergic synapse in the mammalian auditory brainstem, which due to its size and accessibility allows direct patch-clamp recordings from the nerve terminal and its postsynaptic target, the principal neurons of the medial nucleus of the trapezoid body (MNTB) (Forsythe 1994). Using whole cell patch-clamp, Inchauspe et al. (2010) showed that the presynaptic Ca^{2+} current-voltage (I-V) relationship is shifted to more hyperpolarizing potentials in R192Q KI calyces, with a maximum current at −20 mV (vs −15 mV in wild-type-WT), with similar reversal potential around 55–60 mV. Maximum presynaptic Ca^{2+} current (I_{pCa}) amplitudes were not significantly different (Inchauspe et al. 2010). Activation curves obtained from the peak amplitudes of tail currents also showed a −6.5 mV shift towards hyperpolarized potentials in KI compared to WT mice while half-inactivation voltages of steady-state inactivation curves were significantly more negative for R192Q KI compared to WT mice. In conclusion, R192Q KI mutation did affect the biophysical properties of presynaptic Ca^{2+} currents (I_{pCa}) where Ca^{2+} channels are opened at more hyperpolarizing membrane potentials.

Assuming that the kinetics of I_{pCa} can be modeled by Hodgkin/Huxley equations, a shift to more negative activation voltages should generate a larger Ca^{2+} current during an AP (Borst and Sakmann 1999). Nevertheless, I_{pCa} evoked by real APs in KI and WT calyx of Held presynaptic terminals were similar in amplitude and kinetic parameters, indicating that the negative shift in activation of presynaptic Ca^{2+} channels in R192Q KI mice had little effect on Ca^{2+} currents evoked by the calyx of Held APs (Inchauspe et al. 2010) (Fig. 12.1 upper panel). As expected, synaptic transmission was not affected at low frequency stimulation in physiological extracellular Ca^{2+} concentration. However clear differences were observed when

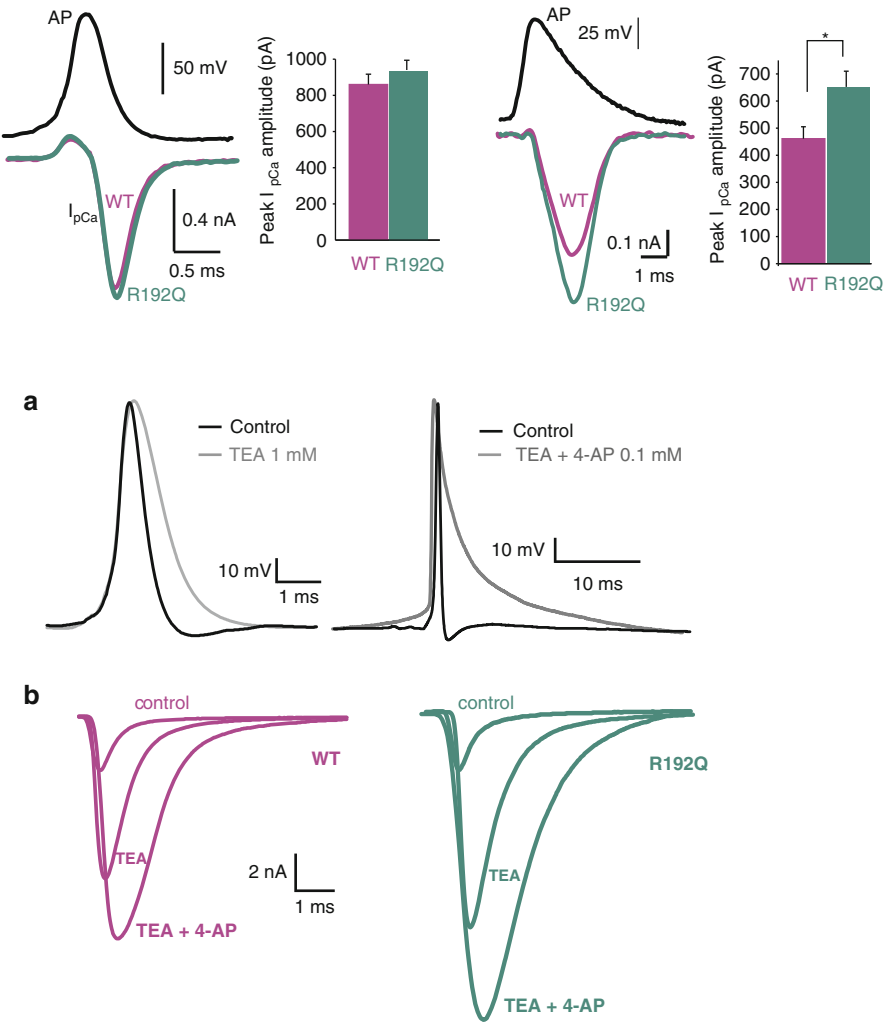

Fig. 12.1 Increasing action potential (AP) duration reveals a gain-of-function in the KI mice. *Upper panel*: AP-evoked P/Q-type Ca^{2+} currents in layer 2/3 pyramidal cells (PC) from WT and KI cortical slices. AP waveforms (*black top traces*) and their corresponding Ca^{2+} current elicited by the above APs in the same cells (*purple* for WT and *blue* for R192Q KI mice). A significant larger Ca^{2+} current was recorded when elicited by a broad action potential in the KI- R192Q neurons (* P = 0.01) (Modified from Inchauspe et al. 2010). *Lower panel:* (**a**) *Upper traces*. Voltage-dependent potassium channel blockers slow presynaptic AP decay during whole cell current clamp recordings at the calyx of Held. After adding 1 mM of TEA halfwidth AP duration increased over 100 % and 500 % after the additional application of 4-AP. Similar changes in action potential duration were recorded in the KI mice calyx of Held. (**b**) *Lower traces* show representative EPSCs in control conditions and after the sequential addition of TEA and 4-AP for WT (*left*) and R192Q KI (*right*) mice, at Ca^{2+} and Mg^{2+} concentrations of 0.75 mM and 2 mM, respectively (i.e., to avoid saturating vesicle release after TEA and 4-AP bath application). A larger increase in the KI EPSCs was observed by increasing AP duration as expected from the gain-of-function observed in the KI Ca^{2+} currents as shown in the *upper panel* (Modified from Inchauspe et al. 2012)

I_{pCa} were evoked by prolonged AP waveforms (e.g., like the APs recorded from pyramidal cells). Triggering Ca^{2+} currents with AP waveforms of increasing duration generate a larger increase in Ca^{2+} currents in the mutated calyx compared to the WT. Accordingly, larger increases in EPSC amplitude and charge were recorded in the R192Q calyx of Held when glutamatergic EPSCs were evoked by broadened presynaptic APs when inhibiting K^+ channels (Inchauspe et al. 2012) (Fig. 12.1 lower panel).

Inchauspe et al. (2010) also showed that $Ca_V2.1$ Ca^{2+} channels in cortical layer 2/3 pyramidal cells (PCs) from KI mice also activated at more negative potentials. PCs had APs with longer durations and smaller amplitudes than those of the calyx of Held. When Ca^{2+} currents (I_{Ca}) from PCs were evoked by APs previously registered in the same cells, KI mice showed an increase in I_{Ca} amplitudes compared to WT mice. In contrast, when I_{Ca} was evoked in PCs by calyx of Held AP waveforms, no amplitude differences were observed between WT and KI mice. These results suggest that longer time courses of pyramidal APs were a key factor for the expression of a synaptic "gain of function" in the KI mice and indicate that consequences of FHM1 mutations might vary according to the shape of the APs in charge of triggering synaptic transmission Thus, the differences in AP durations that elicit cortical excitatory and inhibitory synapses may explain the unaltered inhibitory neurotransmission observed by Tottene et al. (2009) at the fast spiking (FS) interneuron- PC synapses as well as the gain-of-function observed at the PC-FS interneuron excitatory synapses, since several types of interneurons and other neurons that display fast spiking behavior have APs with short half-widths durations (Ali et al. 2007), while PCs depict long APs (Fig. 12.2).

Repetitive stimulation of afferent axons to the MNTB at different frequencies causes short term depression of EPSCs that recover significantly faster in R192Q KI than in WT mice. Faster recovery in R192Q KI mice is prevented by the Ca^{2+} chelator EGTA-AM, pointing to enlarged residual Ca^{2+} (i.e., certain $[Ca^{2+}]$ that is hypothesized to remain free in presynaptic terminals between stimuli) as a key factor in accelerating the replenishment of synaptic vesicles (Inchauspe et al. 2012). In this way, fast recovery of vesicle recycling during high frequency transmission can also contribute to the increased excitability in FHM mutant mice. Although an established model that explains migraine attacks is still lacking, a favored hypothesis considers that the abnormal balance of cortical excitation-inhibition and the resulting persistent state of hyperexcitability of neurons in the cerebral cortex may be associated with the increased susceptibility for CSD, which is believed to initiate the attacks of migraine with aura (Lauritzen 1994; Welch 1998).

12.4.2 Ataxia Type 2

Primary episodic ataxias are autosomal dominant channelopathies that manifest as attacks of incoordination and imbalance. Mutations in two genes, KCNA1 (Episodic ataxia type 1, EA1) and CACNA1A, (Episodic ataxia type 2, EA2)

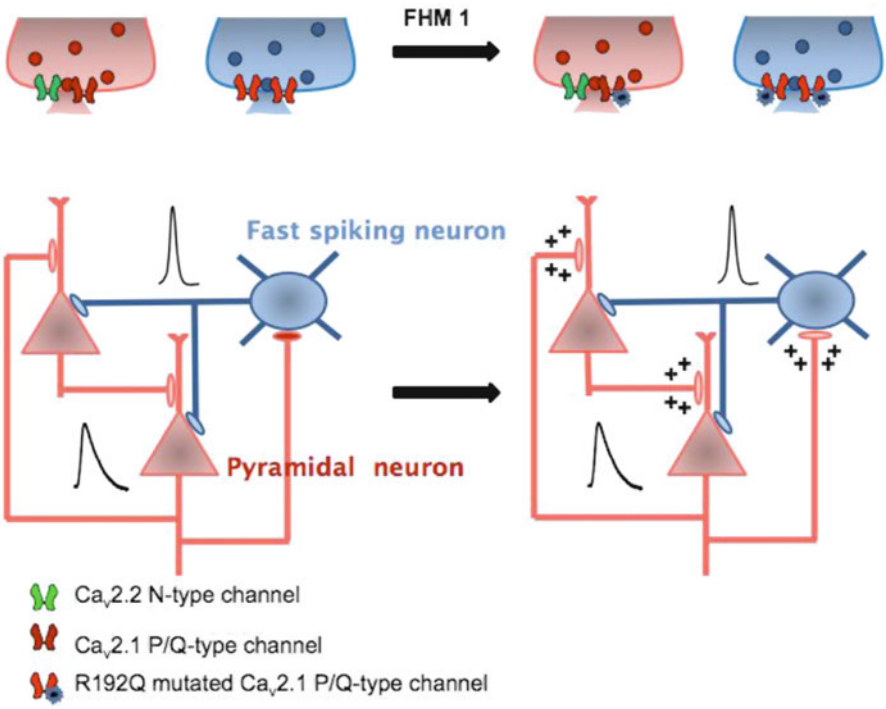

Fig. 12.2 Cortical excitatory and inhibitory synaptic transmission in FHM 1. *Upper panel*: Glutamatergic (*red*) and GABAergic (*blue*) nerve terminals displaying different types of calcium channels in normal mouse and in FHM1 model. *Lower panel*: Cortical circuit involving recurrent excitatory synapses between pyramidal cells (PCs) and reciprocal excitatory and inhibitory synapses between PC and fast spiking interneurons. In FHM1 the broad action potential of PC leads to a gain-of-function of presynaptic CaV2.1 channels and an enhanced action potential-evoked glutamate release. Gain-of-function is not expressed at the inhibitory GABAergic synapses between the short duration AP fast spiking neurons and PCs despite being mediated by CaV2.1 mutated channels. Enhanced glutamatergic release between PCs would increase network excitation. Stronger inhibition is expected from an enhanced recruitment of fast spiking interneurons by the glutamatergic release but may not be enough to keep a normal excitation-inhibition balance thus establishing the basal conditions of increased excitability consistent with the episodic nature of the disease

account for the majority of identified and best characterized cases of episodic ataxia. EA2 is characterized by ataxia, interictal nystagmus and cerebellar atrophy. In some patients, symptoms can be fully controlled with acetazolamide, a carbonic anhydrase inhibitor (Jen et al. 2007). EA2 generally has an onset in the second decade of life and a progressive cerebellar syndrome often appears in later years. Marked central, interictal oculomotor deficits occur in over 90 % of patients (Sasaki et al. 2003; Engel et al. 2004). EA2 is allelic with FHM1 (Ophoff et al. 1996) and, in some families, episodes of both ataxia and hemiplegic migraine occur in the same patients (Ducros et al. 2001; Jen et al. 2004).

There are now more than 80 mutations in CACNA1A identified in individuals with EA2 (Guida et al. 2001; Mantuano et al. 2004; Eunson et al. 2005; Jen et al. 2007; Strupp et al. 2007) and it is likely to expand even further with comprehensive functional characterization of the CACNA1A gene (Veneziano et al. 2009; Mantuano et al. 2010). Most commonly, mutations predicted premature termination of the open reading frame likely subject to nonsense mediated mRNA decay or rapid degradation of truncated protein products. Since transgenic co-expression of mutant plus wild-type $Ca_V2.1$ protein decreases Ca^{2+} current significantly, this EA2-associated truncation clearly has a dominant negative effect (Jeng et al. 2006) by interfering with the correct folding and trafficking of wild-type channels, while causing them to be retained in the endoplasmic reticulum (Page et al. 2004, 2010; Raike et al. 2007; Jeng et al. 2008; Mezghrani et al. 2008; Veneziano et al. 2011). Thus, the likeliest underlying mechanism of these truncating or missense mutations is a major reduction in the number of functional $Ca_V2.1$ channels (Guida et al. 2001). Another hypothesis that provided a novel insight into possible mechanisms of disease in EA2 was that wild-type and mutant P/Q channels competed for channel-type-specific slots in the presynaptic active zone (Cao et al. 2004). This mechanism might contribute to both the dominant mode of inheritance of $Ca_V2.1$ channelopathies and the resulting loss of synaptic efficacy.

Earlier studies have demonstrated the central role of $Ca_V2.1$ Ca^{2+} channels (α1A subunit) in evoked transmitter release at the mammalian motor nerve terminal (Uchitel et al. 1992a; Protti and Uchitel 1993; Katz et al. 1997). Consistent with the expected alterations in expression of this channel in EA2, electromyographic studies in EA2 patients demonstrated a reduced safety factor of neuromuscular transmission and increased jitter (i.e., variance in synaptic transmission delays) as well as blocking on voluntary single fiber electromyography (Jen et al. 2001). In vitro microelectrode studies showed marked reduction of end-plate potential quantal content, confirming a presynaptic defect in neuromuscular transmission (Maselli et al. 2003). Interestingly, the end plate potentials showed high sensitivity to N-type blockade with ω-conotoxin GVIA not seen in controls (Protti and Uchitel 1993). The finding of impaired neuromuscular transmission in EA2 patients is consistent with a loss-of-function mechanism for EA2 mutations. The presence of N-type Ca^{2+} channels in the neuromuscular junction of EA2 patients reflects a possible compensatory mechanism to restore normal activity both at the neuromuscular junction and at central neuronal synapses. Indeed, extensive studies at peripheral and central synapses performed in transgenic mice where the P/Q type Ca^{2+} channel was genetically ablated ($Ca_V2.1$ -/-), provided clear evidence of channel substitution.

Elimination of P/Q Ca^{2+} channels in mice by ablation of the CACNA1A gene induces a progressive neurological deficit about 10 days after birth. The animals start having difficulty walking, absence seizures, ataxia and dystonia. The $Ca_V2.1$ -/- mice deteriorate rapidly, and die at $\sim$3 weeks of age from multiple causes including starvation, but synaptic transmission deficits may play a central role (Jun et al. 1999; Fletcher et al. 2001; Llinas et al. 2007). Although residual synaptic function in $Ca_V2.1$ -/- mice relies on other types of Ca^{2+} channels (Urbano et al. 2003;

Inchauspe et al. 2004, 2007; Pagani et al. 2004), there are clear deficiencies in its dynamics. Indeed, neuromuscular junctions are among the most severely affected synapses since ACh release in these mice depends on both $Ca_V2.2$ and $Ca_V2.3$ channels. Strikingly, though $Ca_V2.2$ are the more abundant channels, $Ca_V2.3$ ones interact more effectively with the exocytotic release machinery. Also, paired-pulse facilitation is almost completely abolished and synaptic synchrony is altered (Urbano et al. 2003, 2008; Depetris et al. 2008). In $Ca_V2.1$-/- mice, Inchauspe et al. (2004) showed partial compensation by $Ca_V2.2$ channels in the calyx of Held. Nevertheless, paired-pulse facilitation of excitatory post-synaptic currents was greatly diminished. In addition, direct recording of presynaptic Ca^{2+} currents revealed that the major functional difference was the absence of activity-dependent presynaptic Ca^{2+} current facilitation.

The question of how the loss of P/Q channel function leads to episodic disturbance of cerebellar function remains unanswered. Although the precise alterations of cerebellar circuitry are unknown, the inhibitory Purkinje cells of the cerebellar cortex are the most likely candidates. The Purkinje cells integrate afferent synaptic input to the cerebellum before relaying the information to the excitatory deep cerebellar nuclei, which in turn communicate with the cerebral cortex and spinal cord. The abundant expression of P/Q channels in Purkinje cells, coupled with evidence of specific degeneration of this cell type in patients with EA2, suggests that a loss (or decline below a critical threshold density) of P/Q channels in Purkinje cells leads to an impaired neurotransmission (Maselli et al. 2003) or abnormal firing patterns in these cells (Walter et al. 2006), with consequences for neuronal and network excitability. In support of this view, mouse models of EA2 (such as the $Ca_V2.1$-/- mouse, and the spontaneous recessive mutations that contribute to various allelic forms of the *tottering* mouse phenotype) exhibit both loss of P/Q channel function in Purkinje cells and deficits in excitatory neurotransmission (Pietrobon 2005).

To test for in vivo effects of ion channel dysfunction on cerebral excitability in patients with EA2, Helmich and colleagues used transcranial magnetic stimulation (TMS) to measure corticomotor excitability (Helmich et al. 2010). TMS is a well recognized method to measure the excitability of corticospinal output neurons and is sensitive to trans-synaptic and intrinsic changes in corticospinal excitability (Kobayashi and Pascual-Leone 2003). This study indicated that patients with EA2 have an excessive increase in motor cortex excitability following a strong facilitatory input which may set the stage for the emergence of paroxysmal neural dysfunction. The abnormal regulation of excitability in EA2 is probably related to dysfunctional $Ca_V2.1$ channels, which is the pathophysiological hallmark of the disease (Pietrobon 2010). Although it is difficult to translate single-cell characteristics into cortical in vivo excitability changes, it is possible to speculate that altered kinetics of the affected $Ca_V2.1$ may lead directly (or through impaired activation of K(Ca) channels) to prolonged synaptic transmission and changes in short term synaptic plasticity (Inchauspe et al. 2007; Catterall and Few 2008) which are crucial for encoding information in neurons (Mochida et al. 2008).

Finally, compensatory reactions to the loss of functional $Ca_V2.1$ Ca^{2+} channels may appear. For example, single-cell recordings in mouse models of EA2 have shown increased Ca^{2+} sensitivity of the intracellular release machinery (Piedras-Renteria et al. 2004) and increased expression of other Ca^{2+} channel subtypes (i.e., N-type Ca^{2+} channels) (Inchauspe et al. 2004). It is likely that these alterations in neurotransmission interfere with the ability of patients with EA2 to regulate the dynamic response to facilitatory input. This could lead to abnormally prolonged neuronal excitability following transient facilitatory events, resulting in the paroxysmal attacks that are characteristic of EA2 (Helmich et al. 2010).

Many spontaneous mutations in mice are also linked to cerebellar ataxia and seizures, and resemble generalized absence epilepsy in humans. They include autosomal recessive CACNA1A mutations in *tottering, leaner, rolling Nagoya, rocker*, and *entla* mice; also mutations in the β4, α2–δ2 and γ2 regulatory subunits in *lethargic, ducky, entla* and *stargazer* strains respectively (reviewed by Pietrobon 2005; Bidaud et al. 2006). Their extensive analysis has been useful in clarifying both the normal physiological role of $Ca_V2.1$ Ca^{2+} channels and how the mutations cause disease.

12.4.3 Spinocerebellar Ataxia Type 6 (SCA6)

SCA6 is a late-onset, progressive, cerebellar syndrome characterized by impaired balance, limb incoordination and dysarthria. SCA6 is an anomaly among the neuronal channelopathies in that the symptoms are generally not episodic but rather slowly progressive. SCA6, originally classified by Zhuchenko et al. (1997), is caused by a CAG repeat expansion in the CACNA1A gene. SCA6 is one of ten polyglutamine-encoding CAG nucleotide repeat expansion disorders comprising other neurodegenerative disorders such as Huntington's disease (Solodkin and Gomez 2012).

It remains controversial whether the mutation exerts neurotoxicity by changing the function of $Ca_V2.1$ channel or through a gain-of-function mechanism associated with accumulation of the expanded polyglutamine protein. Watase et al. (2008) have generated three strains of KI mice carrying normal, expanded, or hyperexpanded CAG repeated tracts in the CACNA1A locus. The mice expressing hyperexpanded polyglutamine developed progressive motor impairment and aggregation of mutant $Ca_V2.1$ channels. Electrophysiological analysis of cerebellar Purkinje cells revealed similar Ca^{2+} channel current density with normal voltage activation and inactivation kinetics in the three KI models, suggesting that expansion of CAG repeats per se does not affect the intrinsic electrophysiological properties of the channels. The pathogenesis of SCA6 is apparently linked to an age-dependent process accompanied by accumulation of mutant $Ca_V2.1$ channels (Watase et al. 2008).

12.5 Autoimmune Disorders Related to $Ca_V2.1$ Ca^{2+} Channels

The autoimmune channelopathies are a group of neurological disorders in which the patient develops raised serum levels of highly specific antibodies against various neuronal or muscle ligand-activated or voltage-activated ion channels or related functional proteins (Buckley and Vincent 2005). The neuromuscular junction (NMJ) is a target of several autoimmune diseases caused by antibodies to pre- or postsynaptic proteins which is facilitated by the relatively easy access of the antibodies into the synaptic cleft where epitopes of the VGCC and nicotinic acetylcholine (ACh) receptors are exposed to the immune system. A body of evidence indicates that the ion channel antibodies are not only markers for an immunotherapy-responsive condition, but pathogenic in themselves.

We will focus on two disorders, Lambert–Eaton myasthenic syndromes (LEMS) and Amyotrophic lateral sclerosis (ALS), where the NMJ is affected by circulating antibodies. In the first case the presence of antibodies against the presynaptic VGCC is well documented. In the second one a growing body of evidence also points to the P/Q VGCCs or related proteins as targets for circulating pathogenic antibodies.

12.5.1 Lambert–Eaton Myasthenic Syndrome

LEMS is a disease of neuromuscular transmission in which autoantibodies against the P/Q-type VGCCs at the presynaptic nerve terminal play a major role in decreasing quantal release of ACh, resulting in skeletal muscle weakness and autonomic symptoms. It is associated with cancer, particularly small-cell lung carcinoma, in 50–60 % of patients. The nerve terminal and carcinoma cells apparently share a common antigen (VGCC), suggesting an immunological cross-reactivity that may lead to the neurological abnormality (for detailed review of clinical features of the disease consult Titulaer et al. 2011). The antibodies are found in over 85 % of patients with LEMS, and they are missing in healthy individuals (Lennon et al. 1995; Motomura et al. 1995).

The pathogenic role of the antibodies was demonstrated by passive transfer to mice of defects in neuromuscular transmission (Lang et al. 1983) and autonomic changes (Waterman et al. 1997).The antibodies may to some degree bind also to other VGCC subtypes, especially to $Ca_V2.2$. However, the primary target and the cause of the down-regulation is their binding to $Ca_V2.1$ (Verschuuren et al. 1998). Antibodies against VGCCs cause their aggregation and internalization, thereby reducing the number of functional P/Q-type channels on the presynaptic motor nerve terminal at the NMJ (Mareska and Gutmann 2004), which in turn leads to a reduced action-potential-dependent ACh release from the motor nerve terminal.

During high-frequency repetitive firing, or following exercise, Ca^{2+} accumulates in the motor nerve terminal, leading to increased ACh release, which explains the post-tetanic potentiation seen during electromyography.

Intracellular recordings from biopsied LEMS muscle show electrophysiological characteristics consistent with those found with electromyography. In response to single stimuli, end plate potentials (EPPs) amplitudes are consistently reduced, but they again increase progressively in size after high-frequency nerve stimulation. In contrast, miniature EPP amplitude rise or decay times are not appreciably changed, demonstrating that neither the size/loading of synaptic vesicles nor the postsynaptic sensitivity to released ACh are affected (Elmqvist and Lambert 1968; Lambert and Elmqvist 1971; Cull-Candy et al. 1980). An interesting feature of neuromuscular transmission in LEMS is the reduction in extracellular $[Ca^{2+}]$-dependence of transmitter release (Cull-Candy et al. 1980). This deficiency may be related to the active zone disorganization altering the distance between the Ca^{2+} channels and the synaptic vesicle, as is also seen in developing NMT (Rosato Siri and Uchitel 1999) and in $Ca_V2.1$ -/- mice (Urbano et al. 2003; Inchauspe et al. 2004, 2007). There is a partial compensation for the low efficacy of Ca^{2+} influx in vesicle release by the accumulation of intracellular Ca^{2+} observed during high frequency stimulation (Piedras-Renteria et al. 2004). In mice chronically injected with LEMS plasma, serum or IgG (and thus depleted of P/Q type VGCC), the normally minor contribution of Ca_V1, L-type, DHP-sensitive VGCC to ACh release assumes greater importance (Flink and Atchison 2003), as previously noted when phosphatases were inhibited (Urbano et al. 2001). However, the putative common mechanisms mediating this 'diversion' of DHP-sensitive VDCC are still unknown.

A similar effect on the VGCCs channel profile was observed in the passive transfer model of LEMS where the ω-agatoxin IVA-sensitive component of neuromuscular transmission was substantially reduced, with a concomitant increase in the effect of N-type and L-type channels blockers (Giovannini et al. 2002). This plasticity in VGCC expression after pathological insult might partly explain why VGCC antibodies do not have a more devastating effect and why there might be phenotypic differences between affected tissues and between individual patients. In agreement with the reduced quantal content release of ACh, the patients have a positive clinical effect when treated with 3,4 diaminopyridine (Sedehizadeh et al. 2011). This compound blocks presynaptic voltage-gated potassium channels while acting as an agonist for VGCCs, thus enhancing the influx of Ca^{2+} into the nerve terminal and the amount of ACh released. Some patients with LEMS and lung tumors develop a cerebellar ataxia syndrome known as "Paraneoplastic cerebellar degeneration" (Graus et al. 2002). P/Q-type VGCCs are present in the Purkinje cells and the LEMS IgG reduces their currents (Pinto et al. 1998). As the cerebellar symptoms in these patients do not usually improve with immunological treatment, their presence is probably a marker for the paraneoplastic condition, and does not define an antibody-mediated disease (Buckley and Vincent 2005).

12.5.2 Amyotrophic Lateral Sclerosis: An Autoimmune Disease Against P/Q-Type Channels?

ALS is a neurodegenerative disorder characterized by progressive neuromuscular dysfunction with a decrease in the number of upper and lower motoneurons (Mulder 1982). Clinical manifestations include fatigue, fasciculations, spasticity, hyperreflexia, weakness and muscle atrophy, ultimately leading to paralysis and death (Rowland 1998). Currently, there are no effective treatments to either stop or delay ALS progression. Approximately 10 % of patients present a familial form of the disorder characterized molecularly by underlying mutations in several different genes (Siddique and Ajroud-Driss 2011). The remaining ALS cases (90 %) are sporadic (sALS) and of unknown etiology. Different hypotheses have been proposed to explain sALS pathogenesis (for review see Kiernan et al. 2011).

There is considerable evidence supporting immune-mediated mechanisms in motoneuronal degeneration. First, autoimmune disorders have been demonstrated in ALS patients (Appel et al. 1986) along with serum antibodies against gangliosides (Pestronk et al. 1989), neurofilaments (Couratier et al. 1998) and VGCC (Smith et al. 1992). Second, spinal inflammatory infiltrates have been detected in ALS patients as well as IgGs in the endoplasmic reticulum of spinal and cortical motoneurons (Engelhardt and Appel 1990; Engelhardt et al. 1990; Troost et al. 1990). However, the presence and pathophysiological relevance of autoantibodies remains controversial (Drachman and Kuncl 1989; Drachman et al. 1995; Arsac et al. 1996).

At the NMJ, pre-incubation with ALS-IgGs stimulates spontaneous synaptic activity (Uchitel et al. 1988, 1992b; Pagani et al. 2006), Ca^{2+} influx (Mosier et al. 1995) and signaling pathways leading to Ca^{2+} release from intracellular stores (Pagani et al. 2006). In all of these processes, an involvement of NMJ Ca^{2+} channels has been established. At NMJs, evoked-transmitter release in response to Ca^{2+} influx is mediated mainly by P/Q-type Ca^{2+} channels (Protti et al. 1991; Uchitel et al. 1992a; Katz et al. 1997). The interaction between ALS-IgGs and Ca^{2+} channels has been proposed by us and others (Uchitel et al. 1988; Llinas et al. 1993; Smith et al. 1994; Carter and Mynlieff 2003; Pagani et al. 2006) but it has not been proved so far. Recently the ALS- IgG reactivity effects were studied in transgenic mouse where the α_{1A} subunits of the $Ca_V2.1$ or the $Ca_V2.2$ were genetically ablated (Gonzalez et al. 2011). As in previous studies, the existence of a subpopulation of ALS patients whose IgG fractions induced an increment in spontaneous ACh release was detected. This effect has been postulated to be the consequence of an increased intracellular Ca^{2+} concentration at the nerve terminal, caused by ALS-IgG binding to the presynaptic membrane. Likewise, the same set of patients that produced synaptic potentiation also had IgGs in serum capable of interacting with mouse NMJ as shown by immunohistochemistry techniques. These results published by Gonzalez et al. (2011) reinforced those obtained by Pagani et al. (2006), in which a positive correlation between ALS-IgG binding and electrophysiological effects were reported. Subsequently the same set of negative and positive ALS IgG sera were

applied on muscle end-plates of $Ca_V2.1$ (P/Q- type) or $Ca_V2.2$ (N-type) α_{1A} subunit KO mice and the immunoreactivity and modulation of spontaneous synaptic activity were analyzed. It was observed that the absence of the N-type channel α_{1B} subunits did not produce any changes in the ALS-IgG reactivity. In contrast the absence of the P/Q-type channel α_{1A} subunits produced a significant decrease in ALS-IgG binding to mouse NMJs, as well as a complete suppression of antibody effects on spontaneous acetylcholine release. These results suggest that IgGs from a group of ALS patients would interact with either the α_{1A} subunit itself or with another protein that has a drastically diminished expression in mouse neuromuscular junction as a consequence of deletion of the P/Q-type channel pore-forming subunit, and whose presence is essential for ALS-IgG-induced synaptic modulation. In fact, it is known that these deficient mice have alterations in the expression pattern of other genes besides the α_{1A} sequence (Piedras-Renteria et al. 2004).

On the other hand, an alteration in P/Q-type Ca^{2+} currents by ALS-IgGs is not likely to occur, since it has been found that ALS-IgGs fail to interact with ^{125}I-ω-conotoxin MVIIC-labeled channels (Drachman et al. 1995) while their specific inhibition by ω-agatoxin IVA does not prevent immunoglobulin-induced synaptic potentiation (Pagani et al. 2006). Therefore the results of Gonzalez et al. (2011) add relevant evidence in favor of the autoimmune hypothesis as one of the possible mechanisms contributing to ALS pathology. They also suggest that NMJ antigens recognized by ALS-IgGs require the presence of P/Q-type channels, although the $Ca_V2.1$ subunit itself does not seem to be the major antigen. Proteomic studies revealing the specific interaction of the P/Q-type Ca^{2+} channels with other presynaptic proteins may provide information about the mechanisms of action of antibodies in ALS patients and how the disruption of active sites by LEMS alters the sensitivity to transmitter release.

12.6 Future Directions

In this chapter, we have provided multiple evidence of the central role of synaptic $Ca_V2.1$ P/Q-type Ca^{2+} channels in certain physiopathological processes characterized by their episodic nature. Although many questions have been answered using in vitro and in vivo approaches with animal models, many more remain to be further solved. Studies should be extended to cortical circuits where the excitation -inhibition balance has been altered and to the conditions where these alterations lead to a disruption in balance triggering the episodic neurological symptoms. In vitro and in vivo analysis of the hyperexcitable cortical circuits may be a useful model to investigate the effect of many drugs empirically used in these episodic disturbances and will certainly help in the design of future therapeutic approaches.

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Chapter 13
Splicing and Editing to Customize Ca_V Channel Structures for Optimal Neural Function

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Abstract Post-transcriptional modification (PTM) including mechanisms such as alternative splicing and A-to-I RNA editing are powerful and versatile mechanisms that greatly expand the coding potential of the genome, giving rise to a more diverse transcriptome and subsequently a larger proteome. While alternative splicing relies on combinatorial assembly of alternative exons, A-to-I RNA enables pin-point recoding of specific single nucleotides in the transcripts. The primary transcripts of neuronal Ca_V channels undergo extensive alternative splicing, but a restricted A-to-I RNA editing, often in a tissue specific manner to generate distinct channel isoforms that could be optimally customized for different aspects of neuronal activities. Here, we discuss the functional relevance of alternative splicing and RNA editing of Ca_V channels focusing on L-type $Ca_V1.2$ and $Ca_V1.3$, P/Q-type $Ca_V2.1$, N-type $Ca_V2.2$ and R-type $Ca_V2.3$ channels.

Keywords Post-transcriptional modification • Alternative splicing • RNA editing • Single Nucleotide Polymorphism • Channelopathy

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13.1 Introduction

Rapid influx of Ca^{2+} through the voltage-gated calcium (Ca_V) channels (VGCCs) initiates a number of physiological processes such as neurotransmitter release and muscle contraction. VGCCs are a group of hetero-oligomeric trans-membrane proteins that are activated upon sensing membrane depolarization. There are ten members of VGCCs that are broadly categorized into two main groups: high-voltage-activated and low-voltage-activated channels. The high-voltage-activated calcium channels can further be subdivided into L-type ($Ca_V1.1$, 1.2, 1.3, and 1.4), P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and R-type ($Ca_V2.3$) channels. The low-voltage-activated T-type channels, on the other hand, consist of the $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ channels. Besides the pore-forming α_1-subunit, auxiliary β, α_2-δ, and/or γ subunits are also required for the formation of functional channels that closely resemble native channels.

Alternative splicing is an exquisite post-transcriptional mechanism to diversify protein structures to expand the range of mammalian physiological processes (Black and Grabowski 2003). The exons of primary RNA transcripts can be assembled in multiple arrays to enable the production of proteomic diversity that possibly confer differences in structure, function, pharmacology, localization and other properties (Black 2003; Matlin et al. 2005). Different mechanisms for alternative splicing exist including utilization of: (i) cassette exon—an alternate exon could either be included or excluded; (ii) mutually exclusive exons—either one of a pair of similar exons is alternatively spliced and retained at a time; (iii) different junctional acceptor or donor splice sites allowing for either the lengthening or shortening of a particular exon; (iv) intron retention where an intron is included in the mature mRNA; and (v) alternative promoter or poly-adenylation sites.

A recent progress in VGCCs is the identification of increasingly more functionally important splice variations of the pore-forming α_1 and auxiliary subunits. The phenotypic variations accompanying the proteomic changes arising from alternative splicing could influence the pharmacological and electrophysiological properties of the VGCCs in the presynaptic terminus of neurons. Moreover, mutations in the pore-forming α_1-subunit were also found to alter the functional properties of VGCCs in the presynaptic terminus. In this review, we highlight five of the seven HVA Ca_V channels, namely the L-type $Ca_V1.2$ and $Ca_V1.3$ channels and the three channels of the Ca_V2 subfamily.

13.2 L-Type $Ca_V1.2$

13.2.1 Functional Roles of $Ca_V1.2$

The $Ca_V1.2$ (α_{1C}) calcium channels were reported to be expressed widely in the soma and proximal dendrites of many types of neurons throughout the central nervous system (CNS) (Westenbroek et al. 1990, 1998; Hell et al. 1993;

Sinnegger-Brauns et al. 2004) and peripheral nervous system (PNS) (Waka et al. 2003). $Ca_V1.2$ channels expressed in hippocampal neurons were involved in post-tetanic potentiation of the GABAergic synapses (Holmgaard et al. 2009; Frey 2010; Malinina et al. 2010). Presynaptic $Ca_V1.2$ channels located on the GABAergic nerve terminals of the medial preoptic nucleus (MPN) neurons are involved in the control of impulse-evoked release and development of synaptic plasticity, which are likely to play a role in the behavioural functions controlled by the MPN (Malinina et al. 2010). The $Ca_V1.2$ channels also mediate cocaine-induced GluA1 trafficking in the nucleus accumbens (Schierberl et al. 2011).

Mice globally lacking the $Ca_V1.2$ L-type calcium channel die in utero before day 15 post-coitum (Seisenberger et al. 2000). Generation of a mouse line with an inactivation of the *CACNA1C* ($Ca_V1.2$) gene specifically in the hippocampus and neocortex ($Ca_V1.2^{HCKO}$) provided a good model for investigating the role of $Ca_V1.2$ channels in the CNS. The report provided strong evidence to indicate that $Ca_V1.2$ channels have an important role in hippocampal long-term potentiation (LTP), a process implicated in the formation of spatial memory of behaving animal (Moosmang et al. 2005; White et al. 2008). Moreover, $Ca_V1.2$ calcium channels have been shown to regulate the presynaptic mechanism of LTP in the amygdala via enhancing glutamate release (Fourcaudot et al. 2009). In another mouse line, deletion of $Ca_V1.2$ channel expression was limited to the anterior cingulate cortex, and these transgenic mice were found to display impaired observational fear learning and reduced behavioral pain responses, demonstrating the role of $Ca_V1.2$ channels in observational social fear (Jeon et al. 2010).

13.2.2 $Ca_V1.2$ Mutation and Single Nucleotide Polymorphism (SNP)

By genome-wide association study (GWAS), two sex-specific SNPs (rs2370419 and rs2470411) were found in *CACNA1C*, the gene that codes for the $Ca_V1.2$ channel, to be associated with mood disorders (Dao et al. 2010). The SNP rs1006737, located in the third intron of the *CACNA1C* gene, was found to be strongly linked to bipolar disorder (BPD) and schizophrenia (Sklar et al. 2008; Nyegaard et al. 2010). Using other neuroimaging modalities such as fMRI, BPD patients with the *CACNA1C* rs1006737 SNP showed higher brain activities in the prefrontal cortex (executive cognition) and hippocampus (emotional processing) (Bigos et al. 2010) and possibly displayed attention deficits (Thimm et al. 2011). To understand how a SNP in intron 3 could be implicated in BPD, it was shown that the occurrence of rs1006737 SNP resulted in a higher expression of the $Ca_V1.2$ transcripts that is assumed to result in correspondingly higher level of expression of the proteins, with presumably larger Ca^{2+} influx in at least the prefrontal cortex and hippocampus (Bigos et al. 2010). However, the pathomechanisms linking the presence of these *CACNA1C* SNPs in patients to disease phenotypes are still largely unknown.

One *de novo* missense mutation G406R in mutually exclusive exons 8/8a of the *CACNA1C* gene is associated with Timothy syndrome (TS) and autism spectrum disorder (ASD) (Splawski et al. 2004, 2005; Bader et al. 2011). The G406R mutation selectively slowed $Ca_V1.2$ channel inactivation upon co-expression with the brain β_1-subunit in Chinese hamster ovary cells (Barrett and Tsien 2008). The severity of the G406R mutation upon disease presentation, such as cardiac arrhythmia, is exon-specific and depended largely on the levels of expression of exons 8/8a in the heart. It will be of interest to determine whether the expression of autistic traits or $Ca_V1.2$-dependent LTP associated with G406R mutation in the *CACNA1C* is similarly modulated. A mouse model of TS (more severe TS2) showed some aspects of autistic spectrum disorder only in the heterozygote TS2-neo mice as the TS2-like heterozygous and homozygous mice died before weaning (Bader et al. 2011). Other mutations, A39V, G402S and G490R, of the *CACNA1C* gene, were also shown to be associated with TS (Liao and Soong 2010).

The IQ-domain of the $Ca_V1.2$ channels, encoded by amino acids 1,624–1,635 of the C-terminus, can be bound by calmodulin (CaM), a Ca^{2+} sensor which mediates Ca^{2+}-dependent inactivation (CDI) and facilitation of the channel. In particular, I1624 of the isoleucine and glutamine (I-Q) dipeptide is essential for CaM binding. Artificially engineered mutation of I1624 substantially attenuates CDI. (Zuhlke et al. 2000). Interestingly, the $Ca_V1.2$ currents of a transgenic knock-in mouse $Ca_V1.2^{I1642E}$ channels showed a modified steady-state inactivation and recovery from inactivation, and an almost abolished voltage-dependent facilitation, indicating that the I/E mutation abolished Ca^{2+}/calmodulin-dependent regulation of the $Ca_V1.2^{I1642E}$ channels (Poomvanicha et al. 2011).

13.2.3 Splice Variations of $Ca_V1.2$

The alternative splicing of $Ca_V1.2$ channels has been followed with interest as their antagonists are used in management of cardiovascular disorders. Previously, it has been reported that the gene coding for the α_1-subunit of $Ca_V1.2$ contains at least 55 exons, of which more than 19 exons can be alternatively spliced (Soldatov 1994; Tang et al. 2004) to generate channel variants with altered biophysical and/or pharmacological properties (Liao et al. 2004; Tang et al. 2004; Zhang et al. 2010). However the information regarding the tissue specific expression pattern of the abovementioned splice variants are currently limited. Interestingly, Tang et al. reported that Fox proteins including Fox1 and Fox2 can regulate $Ca_V1.2$ exon 9* and exon 33 expression differentially during neuronal development (Tang et al. 2009). The same group also discovered that the polypyrimidine tract-binding protein mediates a switch from exon 8 to 8a during neuronal differentiation (Tang et al. 2011). What could be of scientific or clinical interests are the examination of factors that regulate or modulate Fox protein function and to assess how any dysregulation may affect physiology and disease.

13.3 L-Type $Ca_V1.3$

Among the four L-type channels, $Ca_V1.2$ and $Ca_V1.3$ are ubiquitously expressed in the central nervous system. However, the lack of a highly selective blocker towards the Ca_V1 channels has hampered the understanding of their respective physiological roles. Nonetheless, extensive studies have suggested that, as compared to $Ca_V1.2$, $Ca_V1.3$ channels play a more significant role in gating low-threshold-activating Ca^{2+} current that underlies neuronal pacemaking (Pennartz et al. 2002; Chan et al. 2007), excitation-transcription coupling (Zhang et al. 2005, 2006; Wheeler et al. 2008), normal synaptic function (Sinnegger-Brauns et al. 2004; Day et al. 2006), cardiac rhythm (Platzer et al. 2000) and hormone secretion (Marcantoni et al. 2007). Even though the $Ca_V1.3$ channels are also widely expressed in the central nervous system, its expression predominates over $Ca_V1.2$ in certain cells such as the cochlear hair cells, sinoatrial node (SAN) of the heart and neurons in the substantia nigra pars compacta and suprachiasmatic nucleus.

13.3.1 The Functional Roles of $Ca_V1.3$ Inferred from $Ca_V1.3^{-/-}$ Knockout Mice

Much of the knowledge regarding the functional roles of $Ca_V1.3$ has been gained from the characterization of the $Ca_V1.3$ knockout mice (Platzer et al. 2000). The $Ca_V1.3$ channels conduct significant inward current at the operating range of the hair cells of the cochlea and the pacemaking cells in SAN due to their low activation threshold (Koschak et al. 2001; Xu and Lipscombe 2001). Correspondingly, deletion of $Ca_V1.3$ resulted in congenital deafness due to an almost complete absence of Ca^{2+} current in the inner hair cells and degeneration of both the outer and inner hair cells (Platzer et al. 2000). The $Ca_V1.3$ channels are expressed in the ribbon synapse of the hair cells and they play a significant role in triggering glutamate release at the auditory synapse (Brandt et al. 2005). In addition, deletion of $Ca_V1.3$ channels impairs the normal development of the auditory brain stem center. As the phenotype appears even before the onset of hearing (Hirtz et al. 2011; Satheesh et al. 2012), it is therefore suggestive that expression of $Ca_V1.3$ channels is essential for the development of the both peripheral sensory cells and neurons.

Furthermore, $Ca_V1.3^{-/-}$mice exhibit bradycardia as a result of SAN dysfunction (Platzer et al. 2000). More recent reports of the same $Ca_V1.3^{-/-}$mice revealed other subtle phenotypic changes. For example, $Ca_V1.3$ deletion impaired the consolidation of conditioned fear (McKinney and Murphy 2006) due to compromised long term potentiation of the amygdala (McKinney et al. 2009). In line with the findings in $Ca_V1.3^{-/-}$mice, a loss-of-function mutation of human $Ca_V1.3$ was recently characterized in two consanguineous Pakistani families (Baig et al. 2011). The mutation resulted in production of non-conducting $Ca_V1.3$ channels and expectedly

subjects homozygous for such mutations suffered from **s**ino**a**trial **n**ode **d**ysfunction and **d**eafness (SANDD) syndrome (Baig et al. 2011). However, other clinical features in human due to loss of $Ca_V1.3$ current are yet to be characterized.

13.3.2 Unique Biophysical Properties of $Ca_V1.3$ Channels and Modulation

The property of the $Ca_V1.3$ current is defined by its gating mechanisms. While the low activation threshold appears to be an intrinsic property of the $Ca_V1.3$ channels, which is still poorly understood, a variety of feedback mechanisms that inactivate the channel in response to either voltage-induced conformational change (voltage dependent inactivation [VDI]) or elevation of intracellular $[Ca^{2+}]_i$ (CDI) have been well characterized. The process of VDI is initiated by the voltage-dependent conformational rearrangement of voltage-sensing domain comprising S1-to-S4 segments (Swartz 2008) leading to subsequent opening of the S6 gate (Liu et al. 1997; Xie et al. 2005), and finally the occlusion of the gate by the I-II loop in a 'hinge-lid' mechanism. Interestingly, a recently identified "shield' that repels the closure of the channel gate by the I-II loop 'lid' appeared to be a unique feature of the $Ca_V1.3$ channel (Tadross et al. 2008), allowing the channel to remain open despite prolonged activation. In comparison, CDI is a negative feedback mechanism arising from Ca^{2+} influx. Ca^{2+}, when bound to the bi-lobe Ca^{2+} sensor, calmodulin (CaM) that is constitutively tethered to the preIQ-IQ domain of the C-terminus of the channel, trigger a series of conformational changes which lead eventually to channel inactivation (Peterson et al. 1999; Zuhlke et al. 1999; Pitt et al. 2001; Erickson et al. 2003; Mori et al. 2004; Dick et al. 2008). Although the intermediate steps leading to CDI remained elusive, a more recent study indicated that the final stage of CDI involved allosteric regulation of the opening of the S6 gate (Tadross et al. 2008).

Fitting with the diverse functional roles of the channel, the gating of $Ca_V1.3$ channel is often differentially modulated in a tissue-specific manner. The native $Ca_V1.3$ current in pancreatic β-cells and SAN displayed substantial inactivation (Plant 1988; Mangoni et al. 2003) matching the profile of $Ca_V1.3$ channels characterized in heterologous systems (Xu and Lipscombe 2001; Song et al. 2003). In contrast, I_{Ca} recorded from hair cells in cochlea showed little inactivation (Platzer et al. 2000; Song et al. 2003) suitably allowing for persistent cellular activity even in the presence of prolonged sound stimulus (Shen et al. 2006; Yang et al. 2006). Several mechanisms have been proposed to explain the tissue-specific specialization of $Ca_V1.3$ channels. Taking cochlea as an example, selective co-localizations of $Ca_V1.3$ channels with various proteins such as syntaxin, CaBP (Ca^{2+}-binding protein) and Rab3-interacting molecule (RIM) have been proposed to slow down channel inactivation (Song et al. 2003; Yang et al. 2006; Gebhart et al. 2010), although none of them have been conclusively shown in the native system. Alternatively, study by Shen et al. (2006) identified an outer hair cell

splice variant of $Ca_V1.3$ channels with disrupted IQ domain due to utilization of alternative acceptor splice site and frame-shift of exon 41. As the IQ domain is essential for calmodulin-mediated CDI, dominant expression of such a splice variant selectively in outer hair cell (Shen et al. 2006) therefore partly explained the slow inactivating Ca^{2+} current that was observed. It is thus interesting that tissue selective post-transcriptional modifications, such as alternative splicing and RNA editing could potentially generate channel variants of customized properties to suit different physiological needs.

13.3.3 Extensive Alternative Splicing Patterns in $Ca_V1.3$ Transcripts

The $Ca_V1.3$ channels are subject to extensive alternative splicing and a total of 16 exons have been reported to be alternatively spliced and some of them showed tissue and even species specific distribution. Despite the rich assortment of channel isoforms with possibly different functional characteristics, the functional impact of alternative splicing of the $Ca_V1.3$ transcript is still not fully understood.

Alternative splicing of the amino terminus (N-terminus) was known to affect the current density of $Ca_V1.3$ channels (Klugbauer et al. 2002; Xu et al. 2003). Inclusion of either exon 1a (Hui et al. 1991; Seino et al. 1992; Williams et al. 1992a) or 1b (Klugbauer et al. 2002) has been reported in mouse. Exon 1b appears to be mouse specific, while in rat and human, exon 1a is constitutively expressed. Although both splice variants support functional currents with similar gating properties in heterologous expression system, exon 1a conferred a much larger current density as compared to exon 1b (Klugbauer et al. 2002; Xu et al. 2003).

The IS6, IIIS2 and IVS3 segments of $Ca_V1.3$ are encoded by three pairs of mutually exclusive exons including exons 8/8a, 22/22a and 31/31a respectively. Interestingly, $Ca_V1.2$ channels display the same splicing patterns in the abovementioned regions and relatively high sequence conservation was observed between $Ca_V1.3$ and $Ca_V1.2$ channels in these three pairs of mutually exclusive exons. The alternative splicing in IS6, IIIS2 and IVS3 segments of $Ca_V1.2$ was known to alter the sensitivity of the channels towards DHP inhibition with exons 8, 22 and 31 conferring higher drug sensitivity (Liao et al. 2005). In contrast, the functional impacts these three pairs of mutually exclusive exons have on $Ca_V1.3$ channels are less well known. Interestingly, the insertional mutation that results in loss of function of human $Ca_V1.3$ channel is located in exon 8a (Baig et al. 2011). While dominant in heart tissue, approximately 60 % of the total rat brain $Ca_V1.3$ transcripts contain exon 8a (Huang and Soong, unpublished data). Therefore, understanding the tissue specific expression of exon 8a in different brain tissues could have profound implication for prognosis and possible target treatment of any neurophysiological disorder of patients suffering from SANDD syndrome (Baig et al. 2011). On the other hand, exon 22a of $Ca_V1.3$ appeared to be expressed specifically in the rat organ

of Corti with unknown functional roles (Ramakrishnan et al. 2002). In comparison, exon 22 is constitutively expressed in other tissues. Lastly although exon 31 and 31a in $Ca_V1.3$ are both ubiquitously expressed in the brain, the differences in their properties remain uncharacterized.

The I-II loop region of $Ca_V1.3$ contains three splice variations including alternative exons 9*, 11 and 13. Exon 9* (Ramakrishnan et al. 2002) and 13 (Ihara et al. 1995) were identified in the rat organ of Corti and pancreas, respectively, with uncharacterized functional impact. On the other hand exon 11 is more ubiquitously expressed in brain and pancreas and deletion of exon 11 was found not to affect the channel gating of $Ca_V1.3$ (Xu and Lipscombe 2001). Inclusion of exon 9* introduces 26 amino acids into the I-II loop of the $Ca_V1.3$ channels. Sequence of exon 9* in chicken $Ca_V1.3$ contains a consensus sequence of serine surrounded by four basic amino acid residues and is therefore a potential substrate for protein kinase (Ramakrishnan et al. 2002). In contrast, no such consensus site was found in the exon 9* of rat or human $Ca_V1.3$ (Ramakrishnan et al. 2002).

The alternate exon 32 encodes part of the extracellular loop between IVS3 and IVS4. Inclusion or exclusion of exon 32 in $Ca_V1.3$ channels has no effect on the gating properties of the channel and neither was sensitivity towards nitrendipine significantly changed (Xu and Lipscombe 2001).

The carboxyl-terminus (C-terminus) of $Ca_V1.3$ represents another hotspot of alternative splicing that has been more extensively characterized. Alternative splicing at exon 41 and mutually exclusive exons 42 and 42a has been shown to regulate the CDI of the channel. Truncation of exon 41 (half exon 41) due to the alternative use of splice acceptor site in exon 41 resulted in complete removal of the IQ domain and early termination of the C-terminus (Shen et al. 2006). Although functional current could still be observed, deletion of IQ domain resulted in complete elimination of CDI (Shen et al. 2006). Selective localization of half exon 41 in cochlear outer hair cell (Shen et al. 2006) corroborated the previous observation of slowly inactivating native $Ca_V1.3$ current recorded in hair cells, highlighting the tissue specific role of such splice isoform in supporting the normal function of the cochlea. Moreover, exon 41 could also behave as a cassette exon. The $Ca_V1.3$ transcripts devoid of the entire exon 41 have been reported in both rat and human brain (Tan et al. 2011; Bock et al. 2011). Deletion of exon 41 results in complete elimination of the IQ domain, leading to frame-shifting and early truncation of the C-terminus. Functionally, $Ca_V1.3[\Delta e41]$ shows much lower current density and much attenuated CDI (Tan et al. 2011). Interestingly, a most recent study identified three closely spaced A-to-I RNA editing sites in the mRNA sequence which codes for tetra-peptide 'IQDY' in the IQ domain (Huang et al. 2012) The editing is found to be mediated by ADAR2, a member of the family of enzyme known as **a**denosine **d**eaminase **a**cting on **R**NA (ADAR). Expectedly, codon changes from ATA to ATG, CAG to CGG and TAC to TGC result in corresponding amino acid changes from I to M, Q to R and Y to C, resulting in a total of 8 possible amino acid combinations in the IQ domain. Reassuringly, peptide variants containing different amino acids in the IQ domain were detected using the state-of-art mass spectrometry. Biophysically, amino acid changes in the IQ di-peptide specifically attenuated the kinetic of CDI.

Physiologically, editing in the IQ domain was shown to regulate normal rhythmic firing activity of neurons in suprachiasmatic nucleus, a hypothalamic region well known for its role as the master control of biological clock in the mammalian system. Most importantly, RNA editing in the IQ domain was found selectively in the central nervous system and is conserved across different species from mouse, rat to human (Huang et al. 2012). Taken together, it is amazing that two post-transcriptional mechanisms including alternative splicing and RNA editing converge on a single exon 41 to exert overlapping function of regulating the kinetic of CDI.

Further downstream, alternative use of either exon 42 or 42a gives rise to the long-form (LF) or short-form (SF) $Ca_V1.3$ channels respectively (Singh et al. 2008). The stop codon in exon 42a results in expression of only six amino acids immediately after exon 41 and therefore resulting in the early termination of the C-terminus. Although both variants are ubiquitously expressed in the brain, the LF channels display distinctive properties such as a more depolarized-shift in window current, higher expression, lower current density and significantly diminished CDI (Singh et al. 2008). The attenuated CDI in the long-form was later explained by the presence of the CDI-inhibiting module (ICDI) domain at the distal carboxyl terminal which actively competed with calmodulin for the binding to the IQ domain (Liu et al. 2010). The anchoring of calmodulin to the preIQ-IQ domain is critical for CaM-modulated channel inactivation (Erickson et al. 2003; Van Petegem et al. 2005). The attenuated binding between calmodulin and $Ca_V1.3$ channel therefore results in much slower channel inactivation. Consistently, the absence of ICDI domain in short-form channels due to truncation of the C-terminus leads to fast CDI. Moreover, half truncation of exon 42 due to the alternative use of splice donor site and alternative use of splice acceptor and donor sites within exon 42 both resulted in frame-shifting and pre-mature termination of the C-terminus (Seino et al. 1992; Williams et al. 1992b; Bock et al. 2011; Tan et al. 2011). Expectedly, the exclusion of ICDI domain in such a splice isoform supported rapid CDI that is similar to that observed for the short-form $Ca_V1.3$ channels.

Lastly, deletion of exon 44 and use of splice acceptor site within exon 48 resulted in shortening of the C-terminus but did not result in large truncation of the C-terminus. Interestingly, both $Ca_V1.3[\Delta 44]$ and $Ca_V1.3[48S]$ channels displayed slightly slower CDI as compared to the long-form channel suggesting that inhibition of CDI by the ICDI-domain is length-dependent (Tan et al. 2011).

Apart from regulation of CDI, the truncations of the C-terminus due to half exon 41, inclusion of exon 42a and half exon 42 have additional functional implications. Firstly, early truncation of the C-terminus effectively excludes two consensus sites for PKA activity. The two sites, identified using mass spectrometry, include serine 1,743 and serine 1,816 located in exon 43 (Ramadan et al. 2009). Phosphorylation of $Ca_V1.3$ channels by PKA was known to substantially increase $Ca_V1.3$ current which potentially underlies the sympathetic control of heart rate (Qu et al. 2005). The C-terminal alternative splicing of the $Ca_V1.3$ transcripts, particularly in SAN, could therefore regulate the responsiveness of heart rate to the regulation by activation of β-adrenergic receptors via cAMP-dependent PKA. Secondly, shortening of $Ca_V1.3$ C-terminus omits C-terminal **S**rc **h**omology **3** (SH3)

domain binding motifs and **p**ostsynaptic **d**ensity-95/discs large/**z**ona occludens-1 (PDZ) binding motif which has been shown to be crucial for interaction with the scaffold protein Shank (Zhang et al. 2005). Such interaction results in postsynaptic clustering of long form $Ca_V1.3$ channels and was later found to be important for processes such as $Ca_V1.3$ dependent phosphorylated cAMP response element-binding protein (pCREB) signaling (Zhang et al. 2005) and G-protein modulation of $Ca_V1.3$ channels by D2 dopaminergic and M1 muscarinic receptors (Olson et al. 2005). In addition, the PDZ binding motif of $Ca_V1.3$ channel is also known to interact with PDZ domain containing protein, erbin. The association of erbin or harmonin with long-form $Ca_V1.3$ results in voltage dependent facilitation of the current (Calin-Jageman et al. 2007). However, harmonin reduced significantly the peak $Ca_V1.3$ I_{Ba} currents by reducing surface expression of the channels (Gregory et al. 2011).

13.4 P/Q-Type $Ca_V2.1$

13.4.1 Functional Roles of $Ca_V2.1$

P/Q-type $Ca_V2.1$ ($\alpha 1_A$) calcium channels are expressed at high levels in the cerebellum, particularly in Purkinje neurons and granule cells (Stea et al. 1994; Ludwig et al. 1997; Kulik et al. 2004), with high expression at the γ-aminobutyric acid (GABA)ergic nerve terminal (Kulik et al. 2004). These channels constitute the major pathways for Ca^{2+} entry at the presynaptic terminals to initiate synaptic neurotransmitters release (Lonchamp et al. 2009). They are also found at the somatodendritic postsynaptic regions throughout the mammalian brain and spinal cord. The two different knock-out mouse strains lacking the expression of the $Ca_V2.1$ (α_{1A}) subunit were characterized to exhibit severe phenotypes, including ataxia and dystonia. In the first knock-out line, the mice died 3–4 weeks after birth after displaying problems in motor coordination associated with cerebellar degeneration and defects in synaptic pruning (Jun et al. 1999; Miyazaki et al. 2004). On the other hand, the second knock-out line permitted observation of late-onset cerebellar degeneration, and the neurological deficits appeared prominently about 10 days after birth (Fletcher et al. 2001). Furthermore, in mice lacking the $Ca_V2.1$ subunit, the cerebella were smaller in size than that of wild-type (WT) littermates (Jun et al. 1999; Fletcher et al. 2001). Interestingly, the N-type channels ($Ca_V2.2$) functionally compensated for the absence of P/Q subunits at the calyx of Held and evoked giant synaptic currents in the calyx of Held and medial nucleus of the trapezoid body (MNTB) neurons in the $Ca_V2.1^{-/-}$null mice (Inchauspe et al. 2004). It has also been reported that presynaptic $Ca_V2.1$ calcium channels mediate short-term synaptic plasticity when interrogated in the superior cervical ganglion (SCG) neurons, and this function was regulated by the neuronal Ca^{2+} sensor proteins (Mochida et al. 2008).

13.4.2 Mutations in $Ca_V2.1$

Mutations of the *CACNA1A* gene coding for the $Ca_V2.1$ channel have been identified in humans to be associated with several autosomal dominant neurological defects, such as familial hemiplegic migraine (FHM), episodic ataxia type 2 (EA2), and spinocerebellar ataxia type 6, SCA-6 (Pietrobon 2005; Melzer et al. 2010). Approximately 20 missense mutations (loss-of-function) associated with type-1 familial hemiplegic migraine (FHM-1) have been identified in the *CACNA1A* gene (Ophoff et al. 1996) and these FHM-1 mutations altered the voltage-dependent properties of the neuronal $Ca_V2.1$ channels (Hans et al. 1999b; Adams et al. 2009, 2010). It was found that a knock-in transgenic mouse harbouring the most common FHM-1 mutation R192Q has increased neuronal P/Q-type current and facilitation of induction and propagation of cortical spreading depression (CSD) (Tottene et al. 2009). The R192Q mutation also allowed for faster recovery from synaptic depression in the calyx of Held (Inchauspe et al. 2012). Another mutation located at the first intracellular loop of *CACNA1A* (A454T) does not cause FHM but is associated with the absence of sensorimotor symptoms in a migraine with aura pedigree as these mutant channels showed weakened regulation of VDI by $Ca_V\beta$ subunits and impaired modulation by syntaxin 1A or SNAP-25 (Serra et al. 2010).

Episodic ataxia type 2 (EA2) is an autosomal dominant neurological disorder arising from loss-of-function mutations in the *CACNA1A* gene. A clearly dominant negative effect of EA2 mutations was revealed by co-expression of several EA2 missense and truncation mutants with WT human $Ca_V2.1$ channels in mammalian cells. The co-expression of mutant $Ca_V2.1$ channels led to the retention of the WT $Ca_V2.1$ channels in the endoplasmic reticulum and the reduction of membrane expression of the WT $Ca_V2.1$ channels, resulting in reduced Ca^{2+} currents (Jeng et al. 2008; Mezghrani et al. 2008). The *rolling* mouse Nagoya (RMN) is an ataxic mutant mouse, first described by Oda (Oda 1973), that carries a loss-of-function mutation in the gene encoding the $Ca_V2.1$ channels (Mori et al. 2000). Four other mutant mice exhibiting similar phenotypes are the *tottering*, *leaner*, *rocker* and *tottering-4j* mice (Fletcher et al. 1996; Pietrobon 2010). These homozygous mutant mice exhibited ataxia and increased noradrenaline, dopamine and serotonin concentrations in the RMN cerebellum (Oda 1973; Nakamura et al. 2005), but the 22 month-old heterozygous mice showed age-related emotional changes such as reduced anxiety or reduced depression due to alterations in the serotonin synaptic transmission (Takahashi et al. 2011). It has also been reported that the amplitude of the parallel fiber-mediated EPSC was drastically reduced in adult ataxic *tottering* mice of 28–35 days old (Matsushita et al. 2002). Moreover, in these *tottering* mice the feed-forward inhibition from the thalamus to layer IV neurons of the somatosensory cortex was severely impaired and the impairment of the inhibitory synaptic transmission was correlated with the onset of absence epilepsy (Sasaki et al. 2006).

Spinocerebellar ataxia type-6 (SCA-6) is caused by expansion of polyglutamine (polyQ) repeats in the cytoplasmic C-terminus of the $Ca_V2.1$ channel (Zhuchenko et al. 1997) and in human, this repeat is only present in the terminal alternative exon 47 (Soong et al. 2002). Unaltered intrinsic electrophysiological properties of $Ca_V2.1$ channels were recently confirmed in SCA-6 knock-in mice carrying expanded CAG repeats in the C-terminus of the $Ca_V2.1$ channels, and this mouse with the $Sca6^{84Q}$ mutation developed progressive motor impairment and aggregation because of the accumulation of mutant $Ca_V2.1$ channels in the Purkinje neurons (Watase et al. 2008). However, it is thought the possibility of a direct toxic effect of the polyglutamine repeat on the Purkinje neurons mediated possibly via the aberrant activation of the inositol 1,4,5-trisphosphate receptor type 1 (ITPR1). The binding of the $Ca_V2.1$ polyglutamine repeat to ITPR1 might disrupt the timing of ITPR1-dependent plasticity in cerebellar Purkinje neurons (Matsuyama et al. 1999; Restituito et al. 2000; Schorge et al. 2010). Similarly, knowing the distribution of splice variants and the combinatorial patterns of alternative exons in the $Ca_V2.1$ channels have been helpful in explaining why spinocerebellar ataxia-6 (SCA-6) pathology and phenotypic expression is mainly confined to the cerebellum and not the prefrontal cortex (Tsunemi et al. 2008).

13.4.3 Splice Variations of $Ca_V2.1$

The P- (Llinas et al. 1989) and Q-type (Randall and Tsien 1995) calcium channels were identified as two different currents owing to their distinct gating, pharmacological and modulatory characteristics. However it was later shown that the different properties were actually attributed to alternative splicing at distinct sites within the α_{1A} subunit gene (Bourinet et al. 1999). So far, a total of seven exonic loci of the $Ca_V2.1$ gene have been shown to undergo alternative splicing as revealed by the "transcript-scanning" method (Soong et al. 2002). Notably, part of the F helix of the EF-hand domain is encoded by a pair of mutually exclusive exons 37a/37b. Alternative inclusion of either exon 37a or 37b gives rise to two channel splice variants that differ in sequence within the EF-hand-like domain (commonly known as EFa or EFb respectively) in the α_{1A} subunit (Zhuchenko et al. 1997; Bourinet et al. 1999; Krovetz et al. 2000; Soong et al. 2002). Functionally, the $Ca_V2.1_{EFb}$ channels displayed calcium dependent facilitation (CDF) only in combination with the exclusion of exon 47 and in response to a global rise in Ca^{2+} concentration (Chaudhuri et al. 2004). However, the $Ca_V2.1_{EFa}$ channels supported robust CDF in the presence or absence of exon 47 (Chaudhuri et al. 2004). Moreover, exons 37a/37b were observed to display a developmental switch after 1–2 weeks from a high level of EFb expression to a high level of EFa expression in rodent brains. Unexpectedly, in human, there was a biphasic switch of EFb and EFa expression over development and in adult life. Besides, age and gender bias were also observed in human brain tissues, suggestive of a possible role of these EF-hand splice variants in neurophysiological specialization (Chang et al. 2007). Our unpublished data has

also demonstrated a compartmentalization of the subcellular expression of the EFa and EFb in neurons raising the question of the role of CDF of $Ca_V2.1$channels in short-term synaptic plasticity. In addition, two novel splice sites were discovered within the II-III loop of rat $Ca_V2.1$ channel that encode for the loop region that overlaps with the **syn**aptic **pr**otein **int**eraction (synprint) sites (Spafford and Zamponi 2003). Both of these splice variants lacked substantial portion of the synprint sites and in particular, the splice variant $Ca_V2.1_{\Delta 1}$ has a much lower current density and a marked depolarizing shift in the voltage dependence of inactivation (Rajapaksha et al. 2008).

By cross-linking and immunoprecipitation (CLIP) screening technique (Ule et al. 2003), it was found that binding of splicing factor Nova protein to YCAY motifs in pre-mRNA determines the outcome of splicing (Ule et al. 2006), Specifically, Nova-2 was found to regulate the alternative splicing of $Ca_V2.1$ channel by repressing inclusion of alternative exon 31a, but in contrast enhancing exon 24a inclusion (Allen et al. 2010). Functionally, the inclusion of exon 31a in $Ca_V2.1$ decreases the affinity of ω-agatoxin IVA for the channel ∼10-fold, and slowed channel activation and deactivation kinetics (Bourinet et al. 1999; Hans et al. 1999a). On the other hand it is speculated that the extracellular location of exon 24a might play a role in mediating interactions with extracellular proteins (Allen et al. 2010).

13.5 N-Type $Ca_V2.2$

The neuron-specific N-type calcium channels ($Ca_V2.2$, α_{1B}) play the role to couple action potential excitation with neurotransmitter release (Takahashi and Momiyama 1993; Dunlap et al. 1995; Reuter 1995). The N-type current was identified by its irreversible inhibition by ω-conotoxin GVIA (Catterall et al. 2005) and the extensive expression pattern of the channels in the central nervous system highlighted its importance in neurophysiology (Tanaka et al. 1995).

13.5.1 The Physiological Functions of $Ca_V2.2$ Channels as Indicated by $Ca_V2.2^{-/-}$ Mice

$Ca_V2.2$ knock-out mice displayed hyperactivity and prolonged vigilance state in novel environment and in darkness (Beuckmann et al. 2003). Furthermore, deletion of $Ca_V2.2$ channels results in more aggressive behavior in mice possibly due to increased firing activity of serotonin neurons in the dorsal raphe nucleus as a result of reduced upstream inhibitory neurotransmission (Kim et al. 2009). In addition, deletion of $Ca_V2.2$ channels enhanced ethanol reward while paradoxically reduced excessive ethanol consumption (Newton et al. 2004). Moreover, the channel is known to be important for pain transmission as supported by several previous studies. Firstly, these channels are extensively expressed in the superficial layer of

the dorsal horn and dorsal root ganglion (DRG) which are the main nociceptive areas at the spinal level (Altier and Zamponi 2004; Bell et al. 2004). Secondly, blocker of the N-type current diminishes the release of neuropeptide such as substance P which is intimately involved in nociception (Smith et al. 2002). More directly, knockout of $Ca_V2.2$ in mice model showed reduced threshold for mechanical and thermal pain, attenuated nociceptive response in phase II of formalin test, visceral inflammation pain model and also attenuated nociceptive symptoms in neuropathic pain model (Hatakeyama et al. 2001; Kim et al. 2001; Saegusa et al. 2001).

13.5.2 Alternative Splicing Pattern in $Ca_V2.2$ Transcripts and Related Functions

The $Ca_V2.2$ channel undergoes extensive alternative splicing in at least ten exons giving rise to a large number of possible combinations. Alternative splicing affects many aspects of channel functions including the biophysical properties, synaptic trafficking, surface expression and G-protein mediated inhibition.

In the I-II loop region, the alternative use of 3' splice accepter site allows for inclusion or exclusion of Ala^{415} (Genbank accession no. M92905). Inclusion of Ala^{415} in rat $Ca_V2.2$ channels resulted in a positive shift of activation potential by $\sim$19 mV while the voltage dependent profile of steady-state inactivation was unchanged (Stea et al. 1999).

The II-III loop region of rat $Ca_V2.2$ channel contains over 400 amino acids and a synprint site that plays a role in synaptic targeting of the channel via interaction with synaptic proteins such as syntaxin and SNAP-25 in a Ca^{2+}-dependent manner (Sheng et al. 1994, 1996). Alternative splicing in this region generated channel isoforms with altered biophysical properties and different synaptic targeting patterns. Firstly, cassette exon 18a encodes 21 amino acids at the N-terminal portion of the II-III loop (Pan and Lipscombe 2000). Functionally, inclusion of exon 18a slowed down the inactivation kinetic of the N-type current in response to a train of action potential stimuli (Thaler et al. 2004). Prolonged channel opening could potentially elevate residual pre-synaptic Ca^{2+} concentration that could contribute to some aspects of synaptic enhancement such as facilitation, augmentation and potentiation (Zucker and Regehr 2002). In addition, exon 18a inclusion shifted the voltage-dependent steady-state inactivation profile to more depolarizing potential specifically in the presence of β_{1b} or β_4 subunit (Pan and Lipscombe 2000). However, although overlapping with the synprint site, it is not known if addition of 21 amino acids could affect the synaptic protein interaction. While dominating in the SCG, the expression of transcripts containing exon 18a ($Ca_V2.2$[e8a]) is reduced to around 50 % in DRG, spinal cord and caudal region of the brain and to only 20 % in rostral brain regions such as neocortex, hippocampus and cerebellum (Pan and Lipscombe 2000).

Secondly, two human $Ca_V2.2$ splice variants Δ1 (ΔArg756-Leu1139) and Δ2 (ΔLys737-Ala1001) (refer to GenBank accession number M94172 for numbering) were discovered that lack large part of the II-III loop domain including the synprint site (Kaneko et al. 2002). Biophysically, shortening of II-III loop domain positively shifted the steady-state inactivation profile and led to a faster rate of recovery from inactivation. In addition, $Ca_V2.2[\Delta1]$ variant displayed reduced sensitivity towards inhibition by ω-conotoxin MVIIA and GVIA (Kaneko et al. 2002). More importantly, deletion of the synprint site correlated directly with a drastically reduced normal synaptic targeting of both splice variants (Szabo et al. 2006). The expression of the two splice variants could be observed significantly in fetal brain and various regions of adult brain including thalamus, hippocampus, amygdala and cerebellum (Kaneko et al. 2002).

The IIIS3-IIIS4 region contained cassette exon 24a which encodes the tetra-peptide serine-phenylalanine-methionine-glycine. However inclusion or exclusion of the alternative exon did not appear to affect the activation or inactivation kinetics. Nor did it change the current-voltage (*I-V*) profile of the channel (Stea et al. 1999; Pan and Lipscombe 2000). The $Ca_V2.2$ splice variant containing exon 24a was observed in both rat brain and sympathetic ganglion (Lin et al. 1997).

Exon 31a encodes a di-peptide glutamate-threonine (ET) in the IVS3-IVS4 loop domain. Inclusion of exon 31a slowed down channel activation and potentially resulted in reduced Ca^{2+} influx in response to action potential stimulation, as predicted by *in silico* modeling (Lin et al. 1999). Exon 31a is only selectively expressed in the peripheral nervous system in the DRG and SCG (Lin et al. 1999), suggesting that excitation-secretion coupling in postganglionic synapses expressing $Ca_V2.2[e31a]$ may be less efficient as compared to synapses in the CNS.

The C-terminus of the $Ca_V2.2$ channel is another region that is extensively alternatively spliced. The F-helix of the EF hand domain of rat $Ca_V2.2$ channel is encoded by a pair of mutually exclusive exons 37a and 37b. Although both $Ca_V2.2[e37a]$ and $Ca_V2.2[e37b]$ channels have the same unitary conductance, selective inclusion of e37a enhanced the expression of $Ca_V2.2$ channels and prolonged the channel open duration as revealed by single channel recording (Castiglioni et al. 2006). The higher expression of $Ca_V2.2[e37a]$ channels would be explained by a more recent discovery that $Ca_V2.2[e37a]$ isoform is more resistant towards ubiquitination and subsequent degradation by the proteasome system (Marangoudakis et al. 2012).

As compared to the $Ca_V2.2[e37b]$ which is ubiquitously expressed throughout the nervous system, the transcripts containing exon 37a is only selectively enriched in a subset of capsaicin responsive nociceptive neurons in DRG that mediates pain response to heat stimuli (Bell et al. 2004). Following selective down-regulation of $Ca_V2.2[e37a]$ by small interfering RNA (siRNA) in cultured rat DRG neurons the release of neurotransmitter substance P from the nociceptor was reduced (Altier et al. 2007). Furthermore, in vivo down-regulation of $Ca_V2.2[e37a]$ by siRNA attenuated inflammation or neuropathy induced thermal and mechanical

hyperalgesia (Altier et al. 2007). However, $Ca_V2.2$[e37a] mRNA was also found to be selectively down-regulated in rat model of neuropathic pain induced by spinal nerve ligation (Altier et al. 2007). Adding to the existing complexity, selective inclusion of exon 37a sensitizes the channel towards a novel form of $G_{i/o}$ protein-mediated voltage independent inhibition induced by activation of G-protein coupled $GABA_B$- or μ-opioid receptors (Raingo et al. 2007).

Therefore, alternative inclusion of exon 37a seems to impose opposing effects in regulating $Ca_V2.2$ channel function in pain pathway; on one hand, prolonged Ca^{2+} influx through $Ca_V2.2$[e37a] would enhance neurotransmitter release allowing for effective nociception, while on the other hand, selective down-regulation of e37a containing $Ca_V2.2$ transcripts in the presence of persistent pain stimuli could result in overall reduction in expression level of the channel and during intense neuronal activity, $Ca_V2.2$e[37a] channel is susceptible to $G_{i/o}$-mediated activity independent inhibition following activation of $GABA_B$- or μ-opioid receptors, leading to the net reduction of N-type currents.

Therefore, to directly elucidate the role of $Ca_V2.2$[e37a] isoform in the pain pathway, a mouse model was developed whereby exon 37a was selectively knocked out (Andrade et al. 2010). Surprisingly, as compared to the wildtype mice, elimination of exon 37a did not result in any significant change of N-type current density in the capsaicin responsive DRG neurons, in contrast to the previous observation in transfected cell line or native nociceptors (Bell et al. 2004; Castiglioni et al. 2006), nor was basal thermal nociception affected, indicating that expression of $Ca_V2.2$[e37b] alone could compensate for the loss of $Ca_V2.2$[e37a] in mediating normal pain pathway. However, the extent of voltage independent inhibition of N-type current upon G protein activation was indeed found to be significantly reduced in the absence of exon 37a, correlating directly with reduced efficiency of morphine induced spine level analgesia in response to noxious thermal stimuli (Andrade et al. 2010). Hence, rather than a molecular target to be inhibited for pain management, the expression $Ca_V2.2$[e37a] isoform is required for effective relief of thermal pain by morphine.

The distal C-terminus of $Ca_V2.2$ channel contains PDZ and SH3 domain binding motifs that interact with the modular adaptor protein Mint-1 and CASK respectively (Maximov et al. 1999). The PDZ domain binding sequence was found to be the last four amino acids ‘DHWC’ of the C-terminus and the SH3 binding sequence is a proline rich sequence ‘PQTPLTPRP’ located at a short distance upstream of the PDZ binding motif. Both sequences are encoded by the exon 46; the last exon of $Ca_V2.2$ channel (Lipscombe et al. 2002). Coincidentally, a human $Ca_V2.2$ splice isoform (Williams et al. 1992b) was observed which utilizes an alternative 3’-splice accepter site within exon 46 (Genbank accession no. M94173.1). This type of splicing event resulted in truncation of exon 46 leading to a frame-shift and premature termination of the channel and thus effectively removing the SH3 and PDZ binding motifs. Upon transfection in matured hippocampus neurons cultured at high density, hemagglutinin-tagged $Ca_V2.2$ channels with intact C-terminus were found to be co-localized in axonal synaptic cluster with synapsin, a presynaptic marker and PSD-95, a excitatory postsynaptic marker (Maximov

and Bezprozvanny 2002). In comparison, a splice isoform of $Ca_V2.2$ channel with a truncated C-terminus showed restricted expression in the soma and proximal dendrites (Maximov and Bezprozvanny 2002). Specifically, mutating either the proline rich or the PDZ binding motif significantly reduced the number of axonal synaptic clusterings of $Ca_V2.2$ channels and mutating both sites almost completely abolished the co-localization of the channel with synapsin, which is suggestive that both the SH3 and PDZ binding sites encoded by exon 46 worked synergistically to promote synaptic targeting of the channel (Maximov and Bezprozvanny 2002). Furthermore, expression of a distal C-terminus peptide containing both motifs in cultured hippocampal neurons not only dominantly suppressed the synaptic localization of the channel, but also reduced the efficiency of depolarization induced exocytosis, emphasizing the importance of correct presynaptic targeting of the $Ca_V2.2$ channel in maintaining normal synaptic function.

13.6 R-Type $Ca_V2.3$

The $Ca_V2.3$ (α_{1E}) transcript encodes the R-type calcium channel that has been shown to be insensitive to blockade by the typical antagonists against L-, P/Q- and N-type channels (Soong et al. 1993; Piedras-Renteria and Tsien 1998; Tottene et al. 2000; Wilson et al. 2000). The $Ca_V2.3$ channels were first reported in rabbit and rat brains (Niidome et al. 1992; Soong et al. 1993) and later described in human and mice brains (Schneider et al. 1994; Williams et al. 1994). These channels are widely expressed throughout central nervous system (Soong et al. 1993; Williams et al. 1994). Analysis of $Ca_V2.3$ deficient mice revealed that the $Ca_V2.3$ current accounted for the majority of R-type current in CA1 hippocampal and cortical neurons (Sochivko et al. 2002), amygdala (Lee et al. 2002) and DRG neurons (Yang and Stephens 2009), while only 47 % of R-type current in dentate granule neurons is attributed to $Ca_V2.3$ current (Sochivko et al. 2002).

13.6.1 Diverse Physiological Functions of $Ca_V2.3$ Channels

$Ca_V2.3$ is identified by its specific sensitivity to spider toxin SNX-482 (Newcomb et al. 1998) which has been widely used for determining the physiological roles of the channel. Some studies have suggested that R-type current plays minor roles in mediating fast neurotransmission, pair-pulse facilitation or frequency facilitation as compared to P/Q-type current, possibly due to the more distant localization of the $Ca_V2.3$ channels from the release sites (Wu et al. 1998, 1999; Dietrich et al. 2003). Rather, the $Ca_V2.3$ current is important for accumulation of presynaptic Ca^{2+} that led to a form of presynaptic LTP that is independent of N-methyl-D aspartate-receptor in the mossy fiber synapse in the mouse hippocampus (Breustedt et al. 2003; Dietrich et al. 2003). Secondly, $Ca_V2.3$ channels are implicated in

mediating pain response as they are highly expressed in DRG and dorsal horn of spinal cord and consistently, $Ca_V2.3$ knockout mice displayed attenuated response toward formalin induced somatic nociception (Saegusa et al. 2000). In addition, $Ca_V2.3$ channels have been shown to play a role in nociception during neuropathy caused by partial sciatic nerve ligation in mice (Yang and Stephens 2009). However, it has also been suggested that expression of $Ca_V2.3$ in the periaqueductal gray could mediate the descending anti-nociception pathway (Saegusa et al. 2000). Inhibiting $Ca_V2.3$ channels in different tissues could therefore result in contrasting effects in pain management. Thirdly, $Ca_V2.3$ knockout mice exhibited enhanced fear in open field tests (Saegusa et al. 2000; Lee et al. 2002), emphasizing the important role of $Ca_V2.3$ currents for some aspects of processing of emotional stimuli in brain regions such as amygdala. Most recently, $Ca_V2.3$ channels were found to be important for oscillatory burst firing activity of neurons of the reticular thalamus (RT) that is associated with absence epilepsy (Zaman et al. 2011). Outside the CNS, $Ca_V2.3$ currents played significant roles in hormonal secretion from neuroendocrine cells such as beta cells in the islets of Langerhans (Grabsch et al. 1999; Vajna et al. 2001) and chromaffin cells in the adrenal gland (Albillos et al. 2000). Down-regulation and deletion of $Ca_V2.3$ gene disrupted the glucose induced insulin release and stress induced hyperglycemia (Pereverzev et al. 2002a, c).

13.6.2 Alternative Splicing Pattern in $Ca_V2.3$ Transcripts and Related Functions

The $Ca_V2.3$ transcripts have been shown to be alternatively spliced at three different exon loci, namely exon 19 and exon 20 in the II-III loop and exon 45 in the C-terminus, giving rise to a total of six channel splice variants (Pereverzev et al. 2002b). Alternative inclusion of cassette exon 19 results in addition of 19 amino acids in the II-III loop region (Soong et al. 1993; Schneider et al. 1994; Williams et al. 1994; Mitchell et al. 2002). The selective use of splice donor and receptor sites within exon 20 results in deletion of seven amino acids and such splice variant is only detected in the rabbit (Niidome et al. 1992). Lastly, expression of cassette exon 45 results in inclusion of 43 amino acids in the C-terminus (Soong et al. 1993; Schneider et al. 1994; Williams et al. 1994; Mitchell et al. 2002).

Patch clamp electrophysiological study subsequently revealed that expression of exon 19 slowed down channel inactivation, correlating with faster recovery from inactivation in the presence of extracellular Ca^{2+} as charge carriers, while other properties such as current density, *I-V* relationship, voltage dependent activation and inactivation profiles of the channel remained unchanged (Pereverzev et al. 2002b). Interestingly, a consensus casein kinase I1 phosphorylation site 'SMWE' was detected within exon 19 (Williams et al. 1994) but its functional role has yet to be determined. On the other hand, the presence or absence of seven amino acids in exon 20 and exon 45 did not result in significant change in the biophysical properties

of the channel (Pereverzev et al. 2002b). Lastly, although both $Ca_V2.3$[e45] and $Ca_V2.3$[Δe45] are expressed equally in the mouse brain, $Ca_V2.3$[e45] transcripts were found to be dominant in human cerebellum (Pereverzev et al. 1998). A protein kinase C consensus site has been identified in exon 45 but yet to be verified (Schneider et al. 1994).

More recently, it was found that the two splice variants $Ca_V2.3$[Δe19, e45] and $Ca_V2.3$[Δe19, Δe45] make up all the $Ca_V2.3$ channels in both trigeminal ganglion and DRG neurons, with $Ca_V2.3$[Δe19, e45] being the dominant form in both tissues (Fang et al. 2007, 2010). Specifically, $Ca_V2.3$[Δe19, e45] is preferentially expressed in small nociceptive neurons that are also positive for tyrosine-kinase A (trkA), isolectin B4 (IB4)-negative and transient receptor potential vanilloid 1 (TRPV1)-positive (Fang et al. 2007, 2010). Interestingly, IB4-negative neurons are known to secrete calcitonin gene-related neuropeptide and substance P (Snider and McMahon 1998) and (TRPV1)-positive neurons mediate thermal nociception and inflammatory hyperalgesia (Szallasi and Blumberg 1999). Overlapping expression of channel variants such as $Ca_V2.3$[Δe19, e45] and $Ca_V2.2$[e37a] in TRPV1-positive neurons (Bell et al. 2004) could have similar function in mediating nociception and indeed deletion of $Ca_V2.3$ attenuated somatic inflammatory pain (Saegusa et al. 2000) and similarly, targeting specific splice variant of $Ca_V2.3$ in nociceptors could be a potential therapeutic target in pain management.

13.7 Conclusion

VGCCs are indispensible in many aspects of neuronal activity ranging from neural development, cell excitability, synaptic plasticity, neurotransmitter release to excitation-transcription coupling. It would be unimaginable that to complete such a daunting list of tasks requires only a handful of VGCCs. However, the cellular machinery utilizes powerful post-transcriptional mechanisms including alternative splicing and RNA editing to vastly expand the transcriptome. Here we highlighted how such mechanisms when applied to Ca_V channels generated alternatively spliced or edited variants with overt or subtle alterations in channel properties that are optimized or adapted for different biological niches. Information regarding distribution of patho-physiological specific channel variants not only allows for discovery of useful biomarker but also development of new therapeutic targets. On the other hand, the phenotypic expression of Ca_V channel mutations could be influenced by the backbone combinatorial assortment of alternatively spliced exons within the channels and by where such splice combinations are expressed selectively in different brain regions or neuronal types. In the long-term, the acquisition of knowledge of the dynamic regulation of the inclusion or exclusion of alternatively spliced exons via activation of intrinsic or external stimuli will be a major thrust in the field. Such knowledge will contribute to spatial-temporal expression of splice

variants and will also provide another means to modulate channel function to adapt to pathological conditions. Harnessing next-generation RNA sequencing technology will certainly help towards the better understanding of the extent and physiological and pathological significance of alternative splicing and RNA editing, and hopefully also at the level of the single neuron.

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Chapter 14
Presynaptic Calcium Channels as Drug Targets for Pain

Peter J. Cox and Edward B. Stevens

Abstract A substantial body of evidence (including in vitro and in vivo pharmacology using selective N-type blockers and studies using $Ca_V2.2$ knockout mouse strains) implicates $Ca_V2.2$ as the major presynaptic VDCC underlying glutamate and neuropeptide release from sensory terminals in the dorsal horn of the spinal cord. In addition, data is emerging to support a key role of VDCC alpha2delta ($\alpha_2\delta$) accessory subunits as modulators of presynaptic VDCC function and regulators of synaptic release. The successful use of the $\alpha_2\delta$-1 ligands, gabapentoids, to treat fibromyalgia and diabetic neuropathy and the development of the ω-conopeptide ziconotide, a $Ca_V2.2$ blocker with clinical efficacy demonstrated in a range of chronic pain disorders has established presynaptic VDCCs as key pain drug discovery targets. This chapter will outline current understanding of the role of presynaptic VDCCs in pain signalling, discuss efforts to develop ω-conopeptides and small molecule inhibitors of $Ca_V2.2$ as novel analgesics and review mechanism of action and clinical use of gabapentinoids and ω-conotoxins.

14.1 Introduction

Voltage-gated calcium channels (VGCC) have been implicated in multiple points of the pain pathway, for example, controlling peripheral excitability of sensory afferents, regulating presynaptic release from central terminals of sensory neurons, governing excitability of second order neurons in the dorsal horn and burst activity of thalamic neurons (Table 14.1). However, current marketed pain therapeutics (ziconotide, Neurontin™ and Lyrica™) which target calcium channels act at a single point of intervention, namely presynaptic neurotransmitter release. This chapter will

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Table 14.1 Change in expression of Ca_V subunits in pain models

Ca_V subtype	Ca_V protein	Location in pain pathway	Change in expression in pain models	Selective inhibitors	Role in pain pathway	References
N-type	$Ca_V2.2$	Presynaptic terminals (LI-II) of sensory neurons in dorsal horn	Upregulated in dorsal horn in inflammatory pain	ω-conotoxins	Regulation of glutamate and neuropeptide release from sensory neurons	Westenbroek et al. (1998), Yokoyama et al. (2003), and Lu et al. (2010)
T-type	$Ca_V3.1$	Presynaptic terminals of sensory neurons in dorsal horn		Ethosuximide mibefradil TTA-P2	Sensory neuron excitability	Matthews and Dickenson (2001a), Maeda et al. (2009), and Jacus et al. (2012)
R	$Ca_V2.3$	Dorsal horn (pre- and post-synaptic) and supraspinal	Upregulated in dorsal horn in neuropathic pain	SNX-482	Central sensitization at spinal cord level (possible supraspinal mechanism)	Saegusa et al. (2001) and Matthews et al. (2007)
L	$Ca_V1.2$	Dorsal horn neurons	Upregulated in dorsal horn in neuropathic pain	Dihydro-pyridines	Central sensitization	Fossat et al. (2010)
P/Q	$Ca_V2.1$	Presynaptic terminals (LII–V) Dorsal horn interneurons?		ω-agatoxin IVA	Regulation of glutamate release from sensory neuron	Westenbroek et al. (1998), Heinke et al. (2004), and Jacus et al. (2012)

explore the role of calcium channels in presynaptic release and pain processing, focusing on the specific role of N-type VGCCs and development of ω-conotoxins and small molecule N-type blockers as pain therapeutics. The $\alpha_2\delta$-1 subunit assembles with presynaptic calcium channels and comprises the molecular target of gabapentinoids. The mechanism of action of gabapentinoid interaction with $\alpha_2\delta$-1 resulting in analgesic efficacy will be discussed.

14.2 N-type VGCCs as Pain Targets

N-type calcium channel α1 subunits ($Ca_V2.2$) are widely expressed in the CNS and localized presynaptically in neurons (Westenbroek et al. 1995), where their primary role is controlling neurotransmitter release (Meir et al. 1999). N-type calcium channels are co-localized with substance P and CGRP in presynaptic nerve terminals of afferent sensory neurones of laminae I and II in the dorsal horn of the spinal cord where they play a key role in regulating pain transmission (Westenbroek et al. 1998). The presynaptic role of N-type channels has been demonstrated using both slice electrophysiology and in vivo pain models using the selective N-type blocker, ω-conotoxins. Using whole-cell patch clamp recording of dorsal horn neurons of lamina I and II of rodent spinal cord slices, excitatory postsynaptic currents (EPSCs) in response to dorsal root stimulation of both C-fibres and Aδ fibres show a high level of inhibition using ω-conotoxin GVIA (Bao et al. 1998; Heinke et al. 2011). A role for N-type channel in pain signalling, using intrathecally administered ω-conotoxins has been demonstrating in a range of pain models using behavioural testing or in vivo electrophysiological recordings of dorsal horn neurones in the spinal cord (Chaplan et al. 1994; Matthews and Dickenson 2001b). A recent behavioural study measured the effects of ω-conotoxin MVIIA on tactile allodynia in response to intrathecal PGE2 or NMDA. Allodynia caused by intrathecal PGE2 is presynaptic in origin and responds to intrathecal ω-conotoxin MVIIA, whilst allodynia induced by intrathecal injections of NMDA is postsynaptic in origin and does not respond to ω-conotoxin MVIIA, demonstrating a presynaptic role of N-type channels (Tsukamoto et al. 2010). A direct role for presynaptic N-type channels in regulating neurotransmitter release from sensory neurones has been demonstrated using immunochemical detection of release in isolated nerve preparations (Maggi et al. 1990; Santicioli et al. 1992). A recent study using neurokinin-1 receptor internalization as a marker of substance P release in the dorsal horn has demonstrated a specific role of N-type over L-type and T-type VGCCs in central terminals of peptidergic afferents using intrathecal administration of selective blockers (Takasusuki and Yaksh 2011).

Three different $Ca_V2.2$ knockout mouse lines have been developed. The homozygous knockout mice show mixed responses to different models of acute thermal and mechanical pain with little consensus on effects of $Ca_V2.2$ ablation on acute pain (Hatakeyama et al. 2001; Kim et al. 2001; Saegusa et al. 2001). In addition, two knockout strains showed different responses (no effect or reduced

nociceptive) in a model of visceral pain which measures writhing in response to intraperitoneal injection of acetic acid (Kim et al. 2001; Saegusa et al. 2001). In contrast, there was consensus between the three $Ca_V2.2$ knockout mouse lines with a clear reduction in pain responses in the second phase of the formalin test (which measures central sensitization in response to inflammation caused by intraplantar injection of formalin). There is also evidence for a reduction in hyperalgesia of $Ca_V2.2$ knockouts in chronic pain models, both in the spinal nerve ligation model of neuropathic pain (Saegusa et al. 2001) and complete Freund's adjuvant (CFA) model of chronic inflammatory pain (Abbadie et al. 2010). The differences between knockout mouse strains in the various behavioural pain models has been suggested to be due to either genetic backgrounds of the mice or technical differences in behavioural testing between laboratories (see Saegusa et al. 2002).

Despite the widespread expression of N-type channels in the CNS with a key role in neurotransmitter release, initial knockout mouse phenotyping suggested that CNS functions were (unexpectedly) normal, however, further detailed investigations of the $Ca_V2.2$ knockout mouse have uncovered CNS phenotypes. For example, $Ca_V2.2$ knockout mice display hyperaggressive behaviour in a resident-intruder test associated with an increased firing activity of serotonin neurons in the dorsal raphe (which is known to be associated with aggression), an increased level of the aggression-related hormone, arginine vasopressin in cerebrospinal fluid and increased dopamine levels in hypothalamus (Kim et al. 2009). In addition, $Ca_V2.2$ knockout mice also display hyperactivity associated with increased vigilance during the dark phase and a change in the pattern of REM and NREM sleep during the light phase (Beuckmann et al. 2003). $Ca_V2.2$ knockout mice also display changes in cardiovascular parameters through changes in sympathetic function due to a key role of N-type channels in controlling presynaptic release (Ino et al. 2001).

Another approach to investigating the specific role of $Ca_V2.2$ channels in sensory neurons has been specific targeting of membrane-tethered conotoxin to nociceptors using $Na_V1.8$ driven expression in transgenic mice. This has the advantage that there is no compensatory change in gene expression, as seen in knockout mice. The targeted toxin approach confirms the specific role of presynaptic $Ca_V2.2$ channels in the pathway, as demonstrated in inflammatory and neuropathic pain models (formalin model and chronic constriction model) and, also, overcomes the technical difficulty of intrathecal administration of conotoxins (Auer et al. 2010).

In the inflammatory pain model using intraplantar injection of CFA there was a decrease in current amplitude of high-voltage-activated (HVA) Ca^{2+} currents in small/medium diameter dorsal root ganglion (DRG) neurons, no change in mRNA in DRGs, but an increase in expression of protein in the dorsal horn measured using Western blotting (Lu et al. 2010). In another inflammatory pain model, using intraplantar injection of carrageenan, $Ca_V2.2$ protein expression measured using both Western blotting and immunohistochemistry was increased in DRG and spinal cord (Yokoyama et al. 2003). These data has been interpreted as demonstrating a change in distribution of $Ca_V2.2$ from cell body to central terminals following chronic inflammatory pain (Lu et al. 2010). In rat models of neuropathy, there is

a similar decrease in N-type current amplitude and no change in mRNA levels in small/medium diameter DRG neurons (Abdulla and Smith 2001; Luo et al. 2001; Yusaf et al. 2001); however, there is less consensus on change in protein expression in the dorsal horn. An upregulation of $Ca_V2.2$ has been reported in the chronic constriction injury model (Cizkova et al. 2002), but not in a model of spinal-nerve injury (Luo et al. 2001).

Mutually exclusive alternative splicing within the C-terminus, close to domain IV, S6 of $Ca_V2.2$ results in channels derived from either exons e37a or e37b, which differ by 14 amino acids. The e37a splice variant is mainly expressed in nociceptive neurons and is associated with increased N-type Ca^{2+} channel density (Bell et al. 2004). Knockdown of the e37a variant using siRNA in cultured DRGs was associated with a reduction in substance P release from capsaicin sensitive neurons, whilst siRNA knockdown in vivo (using intrathecal injection) demonstrated that e37a variant had a role in acute thermal pain and mechanical hyperalgesia in inflammatory (inhibition of both phase I and II of the formalin model) and neuropathic (chronic constriction injury) models (Altier et al. 2007). A detailed biophysical analysis of the two splice variants heterologously expressed in tsA201 cells has demonstrated that the C-terminal region encoded by exon 37 regulates channel gating; expression of $Ca_V2.2$ channels from the e37a variant results in a hyperpolarizing shift in voltage-dependence of activation and reduced rate of channel deactivation compared to the e37b varant (Castiglioni et al. 2006). Single-channel analysis showed no change in channel conductance between splice variants, whilst assessment of expression of protein in the membrane using measurement of gating currents demonstrated greater expression of e37a variant compared with e37b variant (Castiglioni et al. 2006). From this biophysical analysis, it can be concluded that increased current densities of e37a variant are due to enhanced expression of this splice variant and shifts in calcium channel gating. The enhanced expression of e37a variant in comparison to e37b variant could be due to differences in ubiquitination of the splice variants (Marangoudakis et al. 2012).

N-type Ca^{2+} channels couple to G-protein coupled receptors (GPCRs), either through a voltage-dependent mechanism via a fast membrane-delimited direct $G\beta\gamma$ interaction or a voltage-independent mechanism involving intracellular signalling pathways such as tyrosine kinase phosphorylation (Weiss 2009). The C-terminal domain encoded by e37a exclusively includes a domain responsible for G protein-dependent inhibition. The G protein-dependent mechanism is voltage-independent, and couples through $G\alpha_{i/o}$ and pp60c-src tyrosine kinase phosphorylation of tyrosine 1747; both $GABA_B$ and μ opioid receptors couple to N-type channels through this G protein-dependent mechanism to regulate $Ca_V2.2$ channel activity (Raingo et al. 2007). In a transgenic mouse model with $Ca_V2.2$ lacking exon 37a there is reduced morphine-induced spinal-level analgesia, as measured by paw withdrawal to noxious heat (whilst in the absence of morphine, acute thermal pain responses were similar to wild-type), demonstrating a loss of μ opioid coupling (Andrade et al. 2010). Y1747, involved in G protein regulation of the e37a splice variant, also contributes to an internalization motif (YXLL). The nociceptin receptor

(ORL1) has also been shown to interact directly with N-type channels, causing internalization through a voltage-dependent and agonist-independent mechanism (Altier et al. 2006).

14.3 Conotoxins as N-type VGCC Blockers

The predatory cone snails (*Conus* spp) use a variety of conopeptides for capturing prey. There are 12 different classes of conopeptide which target voltage-gated channels, ligand-gated channels, GPCRs and monoamine transporters (reviewed by Lewis et al. 2012). The ω-conotoxins family includes over 30 structurally-related toxins from 11 species of cone snail and target N- and P/Q calcium channels. The ω-conotoxins CVID, CVIE, CVIF, GVIA, and MVIIA are selective for $Ca_V2.2$, while MVIIC and MVIID have greater potency against $Ca_V2.1$ than $Ca_V2.2$ (Lewis et al. 2012). The $\alpha_2\delta$ subunit has been shown to be upregulated in a range of models of chronic pain. However, co-expression of $\alpha_2\delta$ subunits with $Ca_V2.2$ causes a decrease in potency of MVIIA, GVIA, CVIB, CVID-F (Berecki et al. 2010; Mould et al. 2004), which might have therapeutic implications for treating chronic pain. The conotoxins differ in rates of recovery from block, where CVIE, CVIF and FVIA demonstrate much faster rates of recovery than the clinical compounds MVIIA and CVID (and could potentially provide a better safety profiles in the clinic) (Berecki et al. 2010; Lee et al. 2010).

Residues in the extracellular S5-P-loop region in domain III of $Ca_V2.2$ have been shown to be critical in binding of ω-conotoxin GVIA (Ellinor et al. 1994; Feng et al. 2001), suggesting that the toxin acts as a pore blocker. This region shows structural similarity to EF-hand motifs suggesting that it has a role in Ca^{2+} permeation. The increase in the rate of recovery from block of ω-conotoxin GVIA in reduced external Ba^{2+} suggests that the EF-hand motif and conotoxin binding site overlap (Liang and Elmslie 2002). Effects of paired combinations of different toxins on N-type channels demonstrated that ω-conotoxin-GVIA, ω-conotoxin MVIIC and ω-agatoxin-IIIA bind to a similar site, distinct from ω-grammotoxin-SIA and ω-agatoxin-IVA which bind to a site associated with channel gating (McDonough et al. 2002). Although the binding site is shared between the different conotoxins, it is not identical, as mutations in S5-P-loop region in domain III have a lesser effect on ω-conotoxin MVIIA than ω-conotoxin GVIA actions (Feng et al. 2003).

The ω-conotoxins form an anti-parallel triple-stranded β sheet/cysteine knot peptide containing six cysteine residues, giving rise to three disulphide bridges and four loop regions. Using a ω-conotoxin GVIA radioligand-binding assay with ω-conotoxin MVIIA/MVIIC loop hybrids, loop 2 and loop 4 were shown to be important for Ca_V subtype selectivity (Nielsen et al. 1999). Extensive alanine substitutions have demonstrated that a variety of different amino acids, which are not always conserved across the different ω-conotoxins, affect potency of $Ca_V2.2$ block. However, the conserved Tyr 13 of loop 2 of ω-conotoxins GVIA and MVIIA

plays a key role in binding to $Ca_V2.2$ (Kim et al. 1995; Schroeder and Lewis 2006). Moreover, amino acid position 10 in ω-conotoxins MVIIA, GVIA and CVID has been shown to influence dissociation rate of the toxin (Mould et al. 2004).

14.4 Conotoxins in the Clinic

Preclinically, ω-conotoxin MVIIA has greater efficacy against chronic pain than acute pain. For example, intrathecal injections of ω-conotoxin MVIIA in rat models of inflammatory pain (carrageenan and CFA) and a variety of rat nerve injury models (spinal nerve ligation, chronic compression injury and partial nerve injury) reversed heat hyperalgesia and mechanical allodynia (reviewed by McGivern 2007). Ziconotide, marketed by Azur Pharma as Prialt, is a synthetic form of ω-conotoxin MVIIA (or SNX111) approved by the US Food and Drug Administration (FDA) in 2004 for treatment of severe chronic pain for patients who cannot use (due to severity of side effects) or do not respond to other analgesics (such as opioids and anticonvulsants) (reviewed by Schmidtko et al. 2010). Intravenous ziconotide administration in humans results in hypotension and bradycardia due to sympatholysis (McGuire et al. 1997), therefore the intrathecal route is the only approved method of administration. Ziconotide is administered by the intrathecal route using either an implanted variable-rate microinfusion device or an external microinfusion device and catheter, giving rise to rapid analgesia through interaction with N-type channels in the dorsal horn of the spinal cord. Intrathecal administration of ziconotide is associated with risks of morbidity and mortality, for example development of meningitis and other infections or catheter- and pump-related complications (Deer et al. 2012). The analgesic efficacy of ziconotide has been demonstrated through three randomised, double-blind, placebo-controlled trials in severe chronic pain patients. Using a rapid intrathecal titration regimen (initial dose of 9.6 μg per day, increasing up to a maximum of 57.6 μg per day within 5–6 days), two clinical studies demonstrated high efficacy of ziconotide compared to placebo in cohorts of patients with severe chronic pain associated with cancer and AIDS (Staats et al. 2004) or severe chronic pain of non-malignant cause (Wallace et al. 2006). However, there were associated serious and persistent CNS-related side effects (such a confusion, delirium, exacerbated depression and suicide risk) some of which resulted in hospital admission. An alternative slow intrathecal titration regime was used in a third clinical study (initial ziconotide dose of 2.4 μg per day, increasing to maximum dose of 21.6 μg per day, within 21 days) of patients with severe chronic pain of multiple causes (Rauck et al. 2006). In this study, CNS-related side-effects were reduced; however, there was also a concomitant reduction in average measures of efficacy in comparison to the fast dosing regimens of the previous clinical trials. However, some individuals with severe chronic pain who were refractory to other analgesics experienced high ziconotide efficacy with the slow dosing regimen. Effective pain relief of ziconotide for a range of chronic pain syndromes has been

reported by several open-label studies (reviewed by McGivern 2007). The FDA and EMEA have approved a slow titration method (similar to that adopted in the clinical trial) up to a maximum ziconotide dose of approximately 20 μg per day for treatment of severe chronic pain.

ω-conotoxin CVID isolated from *Conus catus* has the highest selectivity for N-type over P/Q channels of all the ω-conotoxins (Lewis et al. 2012). Intrathecally administered ω-conotoxin CVID has been demonstrated to have equivalent analgesic efficacy to ω-conotoxin MVIIA, but reduced motor-related side effects at lower doses (Scott et al. 2002). The improvement in therapeutic window of ω-conotoxin CVID over ω-conotoxin MVIIA was not dramatic enough to warrant development of a new therapeutic; synthetic ω-conotoxin CVID, leconotide (CNS004, previously AM336) was originally being developed as an intrathecal monotherapy, but had marked central side effects in a Phase I study (Cousins et al. 2002). Leconotide is now being developed by Relevare Pharmaceuticals as a novel intravenous analgesic in combination with an opioid or non-opioid analgesics. This is based on preclinical data using an intravenous injection of leconotide in combination with intraperitoneal injection of morphine or flupirtine in rat models of bone cancer pain or diabetic neuropathy (Kolosov et al. 2010, 2011) In these studies, there appeared to be additive or synergistic effects of drugs in combination (without causing an increase in side effects). Intravenous administration of ω-conotoxins CVID and MVIIA in rabbit both demonstrated similar sympathetic effects on cardiovascular parameters (fall in blood pressure and tachycardia), however, in contrast to ω-conotoxin MVIIA, ω-conotoxin CVID had mild effects on reflex sympathetic vasoconstrictor responses associated with postural hypotension (Wright et al. 2000a, b). A study comparing intravenous effects of ω-conotoxins MVIIA and CVID on rat cardiovascular parameters reported differential effects on blood pressure: in contrast to ω-conotoxin MVIIA, ω-conotoxin CVID did not cause hypotension at a dose giving analgesia in model of diabetic neuropathy (Kolosov et al. 2010). The difference in effects on sympathetic function could partly be due to differences in selectivity for N-type over P/Q-type channels (Lewis et al. 2012). It has also been postulated that differential effects of intravenous ziconotide and leconotide on sympathetic function are due to lower affinity of leconotide for the peripherally expressed $Ca_V2.2$ splice variant containing exon e31a and/or reduction in potency in the presence of $Ca_V\beta_3$ (Lin et al. 1997; Bell et al. 2004; Lewis et al. 2012). As the mechanism of action of leconotide is block of $Ca_V2.2$ channels within the spinal cord, the analgesic efficacy from intravenous administration is difficult to reconcile with the low blood-brain-barrier permeability of cysteine-knot peptides. However, a pharmacokinetics study with intravenous radiolabelled ziconotide suggested that although CNS exposure was low, the range of concentrations measured was sufficient to cause pharmacological block of N-type channels (Newcomb et al. 2000). Measurement of central exposure of ω-conotoxin CVID following intravenous administration has yet to be reported. Relevare Pharmaceuticals plan to develop a CNSB004 combination therapy for treatment of cancer pain, joint pain and post-operative pain.

14.5 Development of Small Molecules to Target N-type VGCCs

The observed efficacy of intrathecal ziconotide in humans demonstrates that $Ca_V2.2$ is an important regulator of pain transmission and provides an attractive target for development of small molecule blockers. However, CNS-related side effects reported for both ziconotide and the more selective N-type ω-conopeptide leconotide could be problematic for clinical progression of small molecule $Ca_V2.2$ blockers. Therefore, use- and state-dependent blockers (which target the VGCC inactivated state) have been suggested as a mechanism for selectively targeting ectopic activity of injured sensory afferents or spontaneous activity of sensitized uninjured afferents, sparing normal nociception but reducing sympathetic and CNS-related side effects (Winquist et al. 2005). Indeed, the use-dependent Ca_V2 subtype blocker TROX-1 has been reported to have a wider preclinical therapeutic window than ziconotide (Abbadie et al. 2010). However, the concept of injured/sensitized sensory afferents firing at higher rate than sympathetic or central neurones is perhaps simplistic as spontaneously active sensory afferents and normal sympathetic efferents fire at similar frequencies (Macefield et al. 2002; Djouhri et al. 2006; Tseng et al. 2009). In addition, many central neurons, such as those in neocortex, hippocampus, basal ganglia, reticular thalamus, auditory nuclei and medial vestibular nucleus, sustain firing rates in excess of 400 Hz (reviewed by Rudy and McBain 2001). Therefore, the benefits of use-dependent $Ca_V2.2$ blockers await further testing in clinical trials.

A chemically diverse range of small-molecule blockers of $Ca_V2.2$ have been developed by several companies including Purdue, Merck, Abbott, Neuromed and Convergence (reviewed by Yamamoto and Takahara 2009; Bagal et al. 2013) (see Fig. 14.1). Related piperazine amide compounds with $Ca_V2.2$ activity have been progressed to clinical trials by two different companies. A state- and use-dependent blocker of $Ca_V2.2$ originally developed by Neuromed as NMED-160 has now been re-formulated to address solubility and bioavailability issues and has been advanced to Phase II by Zalicus (as Z-160). Preclinically, the orally available compound showed efficacy in rat inflammatory and chronic pain models with heart rate, blood pressure, balance and gait (Snutch et al. 2003). Although NMED-160 shows selectivity for $Ca_V2.2$ over $Ca_V1.2$, it has been reported to have similar potency for $Ca_V2.2$ and $Ca_V3.2$ using both tonic and use-dependent electrophysiological protocols (McNaughton et al. 2008). In comparison, Convergence's state-dependent $Ca_V2.2$ blocker, CNV2197944 has reported selectivity for $Ca_V2.2$ over $Ca_V1.2$, $Ca_V3.2$ and $Ca_V2.1$. CNV2197944 has been shown to have preclinical efficacy in a range of pain models using behavioural and in vivo electrophysiological testing, whilst the presynaptic afferent terminal has been confirmed as the site of drug action using electrophysiological recordings from rat spinal cord slices. CNV2197944 displayed no effect on motor co-ordination, heart beat or blood pressure at higher doses (Morisset et al. 2012). CNV2197944 completed Phase I studies in 2012 which demonstrated dose-proportionate pharmacokinetics and few adverse events.

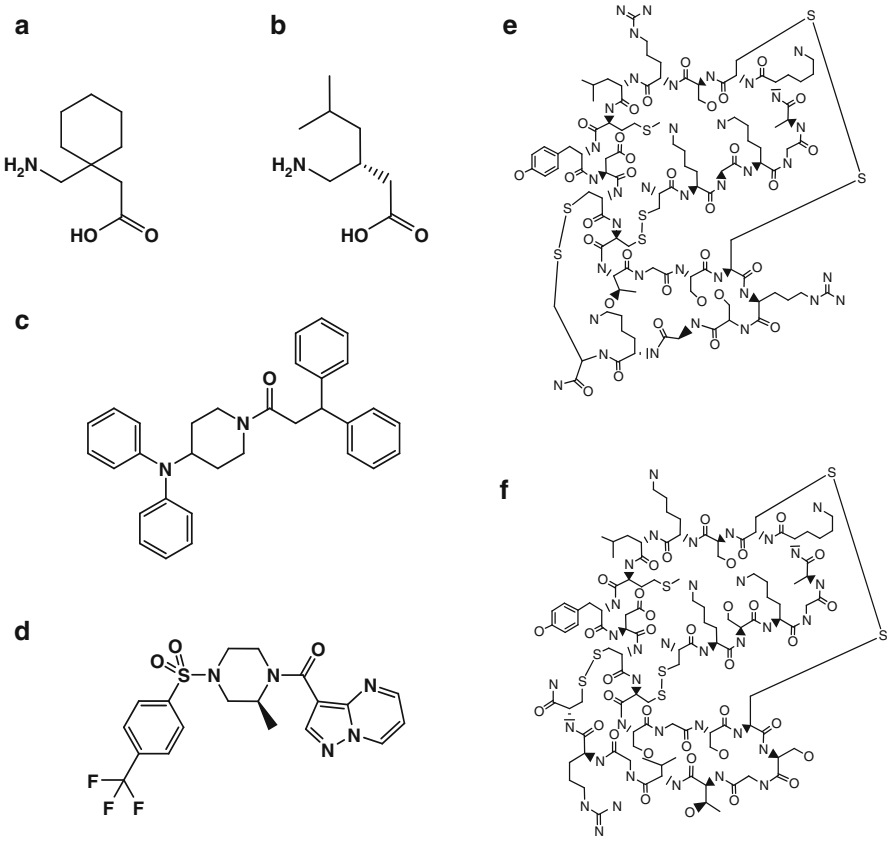

Fig. 14.1 Chemical structures of calcium channels modulators developed as clinical analgesics. Gabapentinoids: (**a**) gabapentin and (**b**) pregabalin. Small molecule $Ca_V2.2$ inhibitors: (**c**) NMED-180 (Z-180) and (**d**) piperazine-sulfonamide published by Convergence (likely similar to CNV2197944). ω-conotoxins: (**e**) ziconotide and (**f**) leconotide

14.6 Other High-Voltage-Activated (HVA) VGCCs as Pain Targets

In addition to N-type VGCCs, L, P/Q and R-type HVA channels have also been shown to be involved in synaptic release of central neurons (Meir et al. 1999) and have been implicated in presynaptic function of sensory neurons terminals in the dorsal horn. P/Q type channels are mainly localized in sensory terminals of laminae II–VI (Westenbroek et al. 1998) and have been shown to play a minor, or even no, role in regulating neurotransmitter release in lamina I neurones using electrophysiological recordings (Heinke et al. 2004; Jacus et al. 2012). However, P/Q channels appear to play a key role in controlling neurotransmitter release of sensory neuron terminals of deeper laminae, as measured pharmacologically

via effects of ω-agatoxin-IVA on spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Jacus et al. 2012). Using the selective blocker SNX-482, R-type channels have been shown to play a role in synaptic release from sensory neurons in the superficial dorsal horn (Jacus et al. 2012). In contrast, the L-type channel is mainly thought to play a postsynaptic role on dorsal neurons (Fossat et al. 2010).

14.7 T-type VGCCs as Pain Targets

Gene knockout (Choi et al. 2007) or knockdown (Bourinet et al. 2005; Messinger et al. 2009) and pharmacological intervention (Todorovic et al. 2001; Nelson et al. 2005; Latham et al. 2009) suggests that the T-type $Ca_V3.2$ subunit may play a role in pain, which was hypothesized to be a largely peripherally mediated effect on neuronal excitability (reviewed in Todorovic and Jevtovic-Todorovic 2011). A number of studies assigned a spinal role for T-type channels in pain neurotransmission since spinal delivery of various blockers, purported to have a VGCC selectivity biased towards T-type, inhibited excitatory neurotransmission in the spinal cord (Matthews and Dickenson 2001a) or pain behaviours (Maeda et al. 2009). However, the non-T-type activity of these compounds at the concentrations used in these studies may confound these conclusions. Compelling evidence of a role for T-type channels in spinal nociceptive neurotransmission was provided in a recent study, which demonstrated that the specific T-type blocker TTA-P2, reduced the frequency, but not the amplitude or decay time of mEPSCs in the superficial lamina of the dorsal horn of the spinal cord in vitro, indicative of a presynaptic site of action (Jacus et al. 2012). The T-type channel subtype responsible for the effects of TTA-P2, which has similar potency against all T-type channels, was confirmed as $Ca_V3.2$, based largely on spinal cord slice recordings from $Ca_V3.2$ knockout animals. The frequency of mEPSCs in spinal cord preparations from knockouts was reduced in comparison to those measured in wild-type animals, and the magnitude of the reduction was similar to the effect of application TTA-P2 to spinal cord slices from wild-type rats (Jacus et al. 2012). Streptozotocin (STZ)-induced nerve injury also increased mEPSC frequency in the superficial dorsal horn of the spinal cord, which was returned to levels seen in uninjured animals by treatment of spinal cord slices from STZ treated animals with TTA-P2. Immunohistochemistry has confirmed expression of $Ca_V3.2$ in the presynaptic termini of non-peptidergic and peptidergic primary afferent neurons in the spinal cord (Jacus et al. 2012).

14.8 Indirect Mechanisms Affecting VGCCs in Pain Therapy

Modulation of presynaptic VGCCs presents an opportunity to develop drugs to treat diseases that result from hyperexcitability of neurons. Directly targeting the pore forming $Ca_V\alpha1$ subunits with small molecules and toxin-based modulators

continues to be exploited as a source of treatments for these diseases. A variety of indirect mechanisms that do not directly target the pore offer additional routes of modulation and, in the case of the gabapentinoids, have led, to the identification of a clinically successful mechanism of modulation.

VGCCs are heteromultimeric assemblies consisting of the pore-forming α1 subunits, and depending on their cell type or tissue specific expression, varied combinations of the auxiliary subunits $\alpha_2\delta$, β and γ subunits. The auxiliary subunits modulate the expression and/or biophysical properties of the channels (reviewed in Dolphin 2009, 2012). However, these subunits would not represent an ideal starting point for the development of drugs since targeting protein-protein interactions is not a facile, or indeed a fruitful, approach in drug discovery, although progress is being made in this area (reviewed in Smith and Gestwicki 2012). It was therefore a somewhat fortuitous discovery that gabapentin (Neurontin), which was originally designed as a gamma amino butyric acid (GABA) mimetic, was shown to interact with the $\alpha_2\delta$ subunit of VGCCs. Gabapentin is widely used as an anticonvulsant and is also prescribed for the treatment of chronic pain. Clinical application of this drug requires careful dose titration since gabapentin has non-linear pharmacokinetics (PK), and sedation and/or somnolence are significant side effects, especially at higher doses. Gabapentin had a 40–50 % success rate in achieving adequate pain relief in clinical trials, but has little effect and/or is not tolerated in an equivalent number of treated individuals. The development of pregabalin (Lyrica) circumvented the issue of non-linear PK, giving more dose titration control to the clinician (reviewed in Bockbrader et al. 2010). Pregabalin is one of the most extensively tested treatments for pain with around 200 studies currently listed on the NIH clinical trials database http://www.clinicaltrials.gov/. A significant number of pivotal trials in neuropathic pain have been conducted with this drug. Despite the success of these gabapentinoid drugs, there is still a significant unmet medical need in chronic pain to improve the number of patients that experience adequate pain relief and develop treatments with reduced side effects.

Although $\alpha_2\delta$ subunits were identified as the molecular targets for gabapentin in 1996 (Gee et al. 1996), the field somewhat struggled to demonstrate any consistent drug effect on VGCC function, despite the high affinity of gabapentin for the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits, and became somewhat side tracked by additional non-$\alpha_2\delta$ mediated mechanisms to explain the action of these drugs (reviewed by Maneuf et al. 2006). There was clear evidence that gabapentin could inhibit neurotransmitter/neuropeptide release from neural tissue in vitro (Maneuf et al. 2001; Maneuf and McKnight 2001; Fehrenbacher et al. 2003) and could modulate evoked presynaptic Ca^{2+} currents (Patel et al. 2000), largely in tissues derived from sensitized animals. It was also apparent that gabapentin (Field et al. 1997a, b) and pregabalin (Field et al. 1999a, b) could affect allodynia and hyperalgesia in rat models of neuropathic pain. However, high concentrations of drug were required to affect endpoints both in vitro and in vivo. In retrospect, this was a clue to the nature of the mechanism, since the concentrations of drug required to affect these endpoints were significantly higher than the affinity of the drug for $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits. This discrepancy was in part explained by the seminal work performed by

Annette Dolphin and co-workers showing that gabapentin inhibited the cell surface trafficking of $\alpha_2\delta$ subunits, which affected Ca_V channel plasma membrane levels, and that inhibition required intracellular access of the drug to its target; therefore, high intracellular gabapentin concentrations were required to elicit effects (Hendrich et al. 2008).

A number of studies demonstrated up-regulation of the $\alpha_2\delta$-1 gene and protein expression in DRGs from nerve injury models (Newton et al. 2001; Abe et al. 2002; Luo et al. 2002; Xiao et al. 2002; Li et al. 2004), which correlated with the onset of allodynia (Luo et al. 2002). Indeed, over expression of $\alpha_2\delta$-1 protein leads to hypersensitivity in genetically modified mice in the absence of nerve injury (Li et al. 2006). The observation that gabapentinoids inhibited subunit trafficking in vitro was extended to a nerve injury model in which it was demonstrated that chronic application of pregabalin reduced $\alpha_2\delta$-1 trafficking to presynaptic sites in the superficial and deeper layers of the spinal cord, without affecting the up-regulation in $\alpha_2\delta$-1 gene or protein expression (Bauer et al. 2009). Moreover, this study presented evidence that it was the directional intracellular trafficking of $\alpha_2\delta$-1 from DRGs to nerve termini in the spinal cord that was affected by drug treatment, providing additional evidence of a role for this protein in trafficking; namely, that ultrastructural immunohistochemical analysis detected $\alpha_2\delta$-1 protein largely in cellular structures important for protein transport such as the endoplasmic reticulum in DRG cell bodies and tubularvesicular structures in dorsal roots (Bauer et al. 2009). More recently it has been shown that $\alpha_2\delta$ regulation of pore-forming subunit trafficking to the cell membrane is a rate limiting step in controlling synaptic release, and that synaptic release is also affected by the $\alpha_2\delta$-$Ca_V\alpha$ subunit interaction (Hoppa et al. 2012); this interaction occurs via a mechanism independent of trafficking that enhances the coupling of VGCC function with synaptic vesicle release. However, in these studies gabapentin had no effect on trafficking or synaptic release. It remains to be determined if the lack of effect of gabapentin relates to the cell type under study.

Gabapentin has also been shown to inhibit synaptogenesis via inhibition of the interaction of $\alpha_2\delta$ subunit with the extracellular matrix proteins thromospondins (TSP) (Eroglu et al. 2009). Inhibition of synaptogenesis seemed to be independent of VGCC function, leading Eroglu et al. to suggest that VGCC function, per se, was not involved in the effects of gabapentin on synapse formation. A specific TSP, TSP-4, may play a role in pain; this protein is upregulated in DRG and spinal cord from the spinal nerve ligation model (Kim et al. 2012). Intrathecal administration of TSP-4 induces a slow onset mechanical hypersensitivity in naive rats, and anti-TSP4 antibody and TSP-4 antisense reverse established neuropathic pain behaviour in the spinal nerve ligation model (Kim et al. 2012). TSPs may have an, as yet, undemonstrated role in modulating calcium channel activity via $\alpha_2\delta$ subunits. Although speculative, there may be a gabapentinoid-sensitive interacting triad between $\alpha_2\delta$s, TSPs and VGCCs to affect presynaptic channel function and modulate pain. The role of a member of this family of extracellular matrix proteins, TSP-1, in the clotting cascade deserves attention as it may provide clues as to the nature of the interaction of these proteins with $\alpha_2\delta$s. The C-terminal globular domain of TSP-1 contains protein disulphide bond reductase activity mediated by

a reactive cysteine residue that breaks disulfide bridges within von Willebrand factor (vWF) multimers, resulting in disulfide bridge formation between TSP-1 and vWF multimers (reviewed in Bonnefoy and Hoylaerts 2008). The TSP-1 vWF multimers are smaller and less active than the homomeric vWF multimers, resulting in regulation of the coagulation cascade. TSP-4 contains a reactive cysteine residue close to, but not at, the same position as in TSP-1. It follows that TSP-4 may interact with the vW factor A (vWA) domain within $\alpha_2\delta$-1 resulting in cleavage of the disulfide bridge between the extracellular α_2 subunit and the membrane associated δ-1 subunit or with another free cysteine residue within $\alpha_2\delta$-1. Intergrin β subunits contain vWA domains that have been shown to interact with extracelluar matrix proteins (Whittaker and Haynes 2002), lending indirect support to this hypothesis. α_2 and δ-2 are encoded by the same gene, and are therefore expressed as a contiguous protein, maturation of $\alpha_2\delta$ proteins involves disulphide bond formation between the α_2 and δ domains, followed by cleavage of these domains that remain linked by a disulphide bond in the mature protein. However, in heterologous cell expression systems, the α_2 and δ-2 subunits of $\alpha_2\delta$-2 largely remain non-cleaved but linked by a disulphide bond, with the exception of lipid rafts in which 50 % of the subunit is appropriately cleaved. In contrast, in the cerebellum the majority of $\alpha_2\delta$-2 is cleaved (Davies et al. 2006). TSP-4 may have a role in the $\alpha_2\delta$ cleavage process in vivo, in either promoting or protecting against cleavage, however this remains to be proven. In the $\alpha_2\delta$-2 protein there are an uneven number of cysteine residues on the exofacial surface suggesting that at least one cysteine residue remains unpaired, and is available for interaction with other proteins (Douglas et al. 2006). It is possible that TSP-4 may interact with $\alpha_2\delta$ proteins via these unpaired cysteine residues to affect cleavage or some other modification of these VGCC auxiliary subunits. At present it is not known what effect uncleaved $\alpha_2\delta$ might have on VGCC function or distribution. The affinity of gabapentinoids for lipid raft preparations containing $\alpha_2\delta$-2 is also higher than for standard membrane preparations and there is evidence to suggest that these compounds may compete with an unidentified endogenous ligand that may bind to the $\alpha_2\delta$ vWA domain (Hendrich et al. 2008).

The vWA domain of $\alpha_2\delta$-1 is essential for trafficking (Cantí et al. 2005), suggesting a link between the proposed mechanism of action of gabapentinoids in inhibiting trafficking of $\alpha_2\delta$s and TSP-4, if this protein interacts with the vWA domain of $\alpha_2\delta$s. However, TSP-4 is an extracellular matrix protein, which likely precludes an effect on intracellular trafficking. It is possible that TSP-4 cleaves $\alpha_2\delta$-1 and becomes linked to the cleaved protein; however discrepancies in the expected molecular weight of the protein have, to date, been largely attributed to glycosylation state and therefore do not support this contention. An extracellular interaction between TSP-4 and $\alpha_2\delta$-1 could enhance VGCC activity or hold the channel at the cell surface/synapse such that recycling is reduced, subsequently leading to increased synaptic activity and pain. Gabapentinoids may disrupt this interaction directly or indirectly by preventing $\alpha_2\delta$ subunits from reaching the cell surface, or by increasing their endocytosis. However, pregabalin had no effect on $\alpha_2\delta$-1 endocytosis in COS-7 cells (Bauer et al. 2009); endocytosis of $\alpha_2\delta$-2 was also reportedly unaltered, whereas forward trafficking of $\alpha_2\delta$-2 from the Rab11

associated endosomes in the endoplasmic reticulum to the plasma membrane was affected by pregabalin (Tran-Van-Minh and Dolphin 2010); this study concludes that inhibition of trafficking to the cell surface, and possibly the presynaptic nerve terminal, was the main effect of chronic drug application. The potential contributions of TSPs were not investigated as these studies predate the discovery of the interaction of $\alpha_2\delta$ subunits with TSPs. It is clear that the gabapentinoids are clinically efficacious drugs, however a deeper understanding of their mechanism(s) of action might reveal new ways to target presynaptic VGCCs via a pathway with clinical validity, however many questions remain to be answered.

TSP-4 has a role in pain (Kim et al. 2012), but it is unknown if other TSP/ $\alpha_2\delta$ interactions might be important under different physiological conditions or diseased states. Likewise, it remains to be determined if interfering with TSP/$\alpha_2\delta$ interactions with gabapentinoids contribute to the sedative effects of these drugs. If not, could this lead to the identification of drugs specifically targeting TSPs with better side effect profiles? This aim could be addressed in part by determining if gabapentinoids retain their sedative effects when administered to TSP knockouts. The rapid onset of effect of gabapentinoids in preclinical pain models is not explained by the relatively slow kinetics of the effects of these drugs on VGCC trafficking in vitro or the effects of chronic drug application on $\alpha_2\delta$-1 trafficking in vivo (Bauer et al. 2009). This may be a reflection of the ability to measure the kinetics of trafficking mechanisms, particularly rapid trafficking within microdomains at the cell surface/synapse. Alternate, as yet unidentified, mechanisms may exist to explain the rapid onset of drug effects, which may be uncovered using in vitro preparations in which rapid effects of gabapentinoids have been measured, such as inhibition of neurotransmitter release (Maneuf et al. 2001; Maneuf and McKnight 2001; Fehrenbacher et al. 2003) and/or potassium evoked presynaptic currents (Patel et al. 2000).

Gabapentinoids bind with high affinity to $\alpha_2\delta$-1 and $\alpha_2\delta$-2, but it was not clear which of these specific subunits may mediate the efficacy and side effects of these drugs; it was hypothesized that, if different drug effects were mediated by separate subunits, improvements to the existing drugs could be achieved through the development of subunit-selective compounds. Compounds that were selective for $\alpha_2\delta$-1 over $\alpha_2\delta$-2 have been identified that were efficacious in pain models but seemed devoid of a sedative effect, as indicated by lack of effect in the rotarod assays (Myatt et al. 2010); however the absence of a sedative effect of the subtype-selective compound used may have been due to the compound achieving ten-fold lower levels in the brain in comparison to plasma, suggesting insufficient CNS penetration. Compounds were identified with high selectivity for $\alpha_2\delta$-1 over $\alpha_2\delta$-2 that had adequate CNS penetration and which were claimed to be non-sedating, however rotarod data was not presented for these compounds. Researchers at Pfizer developed two genetically modified mice that encoded for $\alpha_2\delta$-1 or $\alpha_2\delta$-2 subunits with mutations that significantly reduced the affinity of the gabapentinoids for these subunits (Field et al. 2006; Lotarski et al. 2011). Studies with these models provided clear evidence that both the efficacy in preclinical pain models and the sedation caused by gabapentinoids were mediated entirely by the $\alpha_2\delta$-1 subunit. The mechanism of pain relief seems to be based on modulation of presynaptic

VGCC function at the first synapse in the spinal cord, in addition to reported effects on descending pathway, but it remains to be determined exactly where within the nervous system these drugs act to produce side effects such as sedation, although the $\alpha_2\delta$-1 is present in significant quantities in a variety of brain regions.

In the clinic, approximately 50 % of people do not respond to gabapentinoids or do not tolerate these drugs because of their significant side effects. The variability in tolerability and efficacy of these drugs requires more detailed studies, combining genetic techniques with careful phenotyping of individuals and their drug responsiveness to understand if there are genetic determinants that may form the basis of a strategy to develop new improved drugs directly or indirectly targeting presynaptic VGCCs and these auxiliary subunits. The identification of the interaction of gabapentinoids with $\alpha_2\delta$-1 and $\alpha_2\delta$-2 suggests that it might be possible to develop drugs targeting other $\alpha_2\delta$ subunits. Recently, Costigan and colleagues suggest that $\alpha_2\delta$-3 might also be a target for the treatment of pain and other sensory disorders (Neely et al. 2010). Drosophila and mice homozygote for $\alpha_2\delta$-3 mutations have deficits in heat pain perception and a synonymous single nucleotide polymorphism in the human $\alpha_2\delta$-3 gene is associated with differences in heat pain sensitivity and susceptibility to chronic back pain.

VGCC β and γ subunits are more challenging targets from a drug discovery perspective, but may have some merit given their role in modulating channel function. The $Ca_V\beta_3$ subunit gene and protein was shown to be upregulated in DRG ipsilateral to injury in the spinal nerve ligation model of neuropathic pain (Li et al. 2012). Both the number of $Ca_V\beta_3$ labelled small diameter neurons and the quantity of $Ca_V\beta_3$ present in these neurons were significantly increased following nerve injury. This also correlated with a small, but significant increase in Ca^{2+} current density and a shift in the voltage dependence of activation in the hyperpolarizing direction in small diameter neurons only. Intrathecal delivery of siRNA specific for $Ca_V\beta_3$ returned gene and protein expression to levels similar to those found in uninjured contralateral DRG, normalised VGCC amplitude and kinetics and reversed allodynia in this model (Li et al. 2012). Indirect evidence of a role for the $Ca_V\beta_2$ subunit comes from disparate studies showing that this auxillary subunit rapidly transfers to the cell surface in association with $Ca_V\alpha$ subunits after stimulation of neurones with insulin like growth factor type 1 (IGF-1) (Viard et al. 2004). IGF-1 is released in response to tissue damage and has been shown to mediate hyperalgesia when given exogenously; siRNA knockdown of IGF-1 partially reversed mechanical allodynia following DRG/nerve root compression (Takayama et al. 2011).

Indirect modulation of presynaptic VGCCs is also possible by targeting GPCRs. There is ample evidence that GPCR modulate N-type VGCCs via a direct interaction between the $G\beta\gamma$ subunits and VGCCs (reviewed in Tedford and Zamponi 2006). Examples from the pain arena include the μ opioid receptor (Heinke et al. 2011) and more recently $GABA_B$ receptors (Adams et al. 2012), both of which have been shown to modulate N-type VGCCs. Adams and colleagues have shown that certain conotoxins act via $GABA_B$ receptors to modulate N-type channels in isolated DRG neurons and inhibit allodynia and hyperalgesia in nerve injury models via a

mechanism that is partly Gαi/o dependent, however the exact mechanism and site of action of these toxins remain to be elucidated fully (Callaghan et al. 2008; Klimis et al. 2011).

A more recent and unusual example of modulation of VGCCs by collapsing response mediator protein-2 (CRMP-2) has been demonstrated. These proteins function in growth cone guidance and neurite outgrowth; however proteomics analysis of the N-type interactome suggested an additional function in VGCC regulation (Brittain et al. 2009; Chi et al. 2009). A direct interaction between CRMP-2 and $Ca_V2.2$ has been demonstrated which increases the number of calcium channels in the plasma membrane, and other mechanisms of VGCC regulation by these phosphoproteins have also been put forward (Wang et al. 2010). Cell permeable peptides that inhibit this interaction are efficacious in reducing evoked behaviours in nerve injury models (Brittain et al. 2011).

14.9 Conclusions

There are numerous ways in which presynaptic VGCCs can be modulated; endogenously via direct protein interactions and indirect signalling mechanisms and exogenously via direct interactions of toxins and small molecules with the pore-forming $Ca_V\alpha$ or the auxillary $\alpha_2\delta$-1 subunit (see Fig. 14.2). Exogenous modulation has led to effective treatments for diseases such as chronic pain because hyperexcitability of presynaptic neurons is fundamental to the diseased state;

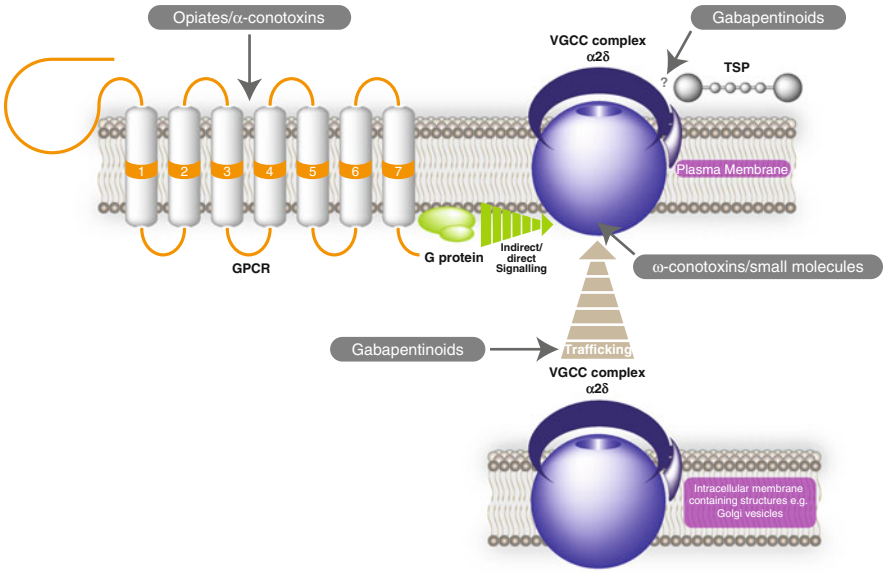

Fig. 14.2 Site of action of exogenous modulation of presynaptic VGCC

however for toxins, at least, their use is severely limited by side effects. This area of drug development avidly awaits the first trials in patients of so-called state-dependent blockers to see if these can deliver on the promise of treatment modalities that target presynaptic VGCCs and possess a large therapeutic window for efficacy over serious side effects. Likewise, despite the success of the gabapentinoids, more work is needed to exploit the compelling indirect mechanisms of altering VGCC function and capitalise on emerging science to develop new drugs, however significant challenges remain, not least proving that these mechanisms are 'druggable' and are clinically as relevant as their predecessors the gabapentinoids.

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Chapter 15
Sensory Pathway Modulation by Calcium Channel $\alpha_2\delta_1$ Subunit

Chunyi Zhou and Z. David Luo

Abstract Voltage-gated calcium channels (VGCC) are importantly involved in modulation of pathophysiological functions, including the transduction of nociceptive and non-nociceptive signals. As an auxiliary subunit of VGCC, the $\alpha_2\delta$ ($Ca_v\alpha_2\delta$) subunit plays critical roles in modulating VGCC expression and functions such as regulations of VGCC trafficking, kinetics of voltage-dependent activation and inactivation. $Ca_v\alpha_2\delta$ also modulates neuronal and synaptic functions through both VGCC-dependent and independent mechanisms. Among $Ca_v\alpha_2\delta_{1-4}$ subunits, $Ca_v\alpha_2\delta_1$ subunit is implicated in pain processing because (1) its upregulation in neuropathic pain models is shown to play a critical role in the onset and maintenance of pain states; (2) its upregulation in sensory neurons leads to dorsal spinal cord neuron sensitization; (3) it is the receptor for gabapentinoids that can normalize activity of sensitized dorsal spinal cord neurons, and have anti-neuropathic pain properties in animal models and patients. In this chapter, we briefly review the regulation of $Ca_v\alpha_2\delta$ and its functional contribution to pathophysiological conditions with a main focus on pain transduction and processing. Underlying mechanisms related to $Ca_v\alpha_2\delta_1$ contributions to pain processing and the therapeutic effects of gabapentinoids are also discussed.

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15.1 Introduction

Ca^{2+} is one of the most important and abundant elements in the body. Membrane depolarization activates voltage-gated calcium channels (VGCC), and causes Ca^{2+} influx, which in turn acts as a second messenger to trigger various intracellular events including enzyme activation, neurotransmitter and hormone release, cell-cell communication, contraction of different kinds of contractile cells, gene expression, cell division, migration and death.

The purified VGCC complex is composed of four subunits, primary channel-forming α_1, auxiliary β, $\alpha_2\delta$, and in some tissue, γ subunits (Takahashi and Catterall 1987; Takahashi et al. 1987; Ertel et al. 2000). Four homologous domains of the α_1 subunit form a Ca^{2+} selective pore. The intracellular β/γ subunits and transmembrane $\alpha_2\delta$ subunit modulate the trafficking and functioning of the VGCC (Felix 1999; Hofmann et al. 1999; Catterall 2000).

Based on membrane potentials required for activation, VGCC were initially divided into high-voltage-activated (HVA) and low-voltage-activated (LVA) channels (Fedulova et al. 1985), then further classified as L-, N- P/Q-, R- and T-type based on their distinct biophysical and pharmacological properties (Nowycky et al. 1985; Dolphin 2006). T-type VGCC have a low-voltage activation threshold, can be activated at the resting membrane potential, thus, contributing to pacemaker activity in excitable cells. Other VGCC have high-voltage activation thresholds, and can be activated at more depolarized membrane potentials. Functional L-, N-, P/Q- and R-type VGCC comprise the principle α_1 subunit, as well as the β and $\alpha_2\delta$ auxiliary subunits in a 1:1:1 stoichiometry. T-type VGCC, on the other hand, appear to require only α_1 subunit for correct function (Bean 1989; Felix 1999; Hofmann et al. 1999; Catterall 2000; Ertel et al. 2000; Dolphin 2006).

The development of selective $Ca_v\alpha_2\delta$ ligands, the gabapentinoids including gabapentin and pregabalin, not only provides us with novel therapeutic agents for neuropathic pain management, but also allows more extensive study of the function of $Ca_v\alpha_2\delta$ at the cellular and molecular level. It is known that $Ca_v\alpha_2\delta$ plays a role in regulating VGCC trafficking to the plasma membrane (Gurnett et al. 1997; Bernstein and Jones 2007), and fine-tuning channel gating properties (Mori et al. 1991; Singer et al. 1991; Klugbauer et al. 1999, 2003; Gao et al. 2000; Davies et al. 2006). There is also emerging evidence suggesting that $Ca_v\alpha_2\delta$ may have functions independent of VGCC. After a brief overview of VGCC subunits, this chapter mainly focuses on structure, cellular/molecular biology and functions of the $Ca_v\alpha_2\delta$ subunit, the mechanisms underlying the action of $Ca_v\alpha_2\delta_1$ proteins on synaptic calcium channel activities, excitatory synaptogenesis that may underlie the mechanism of gabapentinoids in pain modulation.

15.2 Calcium Channel Subunits

The channel forming α_1 subunit ($Ca_v\alpha_1$, 175 kDa) is the principle subunit of VGCC. In mammalian cells, there are ten genes encoding $Ca_v\alpha_1$. Based on amino acid sequence similarity, the $Ca_v\alpha_1$ subunit can be divided into three subfamilies: Ca_v1, Ca_v2, and Ca_v3 (Catterall 2000; Ertel et al. 2000; Arikkath and Campbell 2003), which are classified as L-type ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v1.4$), P/Q-type ($Ca_v2.1$), N-type ($Ca_v2.2$), R-type ($Ca_v2.3$), and T-type ($Ca_v3.1$, $Ca_v3.2$, $Ca_v3.3$) VGCC based on their pharmacology, electrophysiological properties, as well as physiological functions (Hofmann et al. 1999; Catterall 2000; Striessnig and Koschak 2008). Each $Ca_v\alpha_1$ contains four homologous domains connected by cytoplasmic loops. Each domain has six transmembrane segments. There is a pore-forming loop (P-loop) between S5 and S6, which contains a highly conserved, negatively charged amino acid, either glutamate or aspartate, forming a signature locus that is essential for Ca^{2+} selection and conduction (Kim et al. 1993; Kuo and Hess 1993). The S4 segment of each domain that contains positively charged amino acids serves as the voltage sensor for activation and initiation of conformational changes that open the pore. These structural features contribute to VGCC gating, ion selectivity, and permeation. $Ca_v\alpha_1$ also contains the interaction sites for other subunits, VGCC blockers and activators. Although $Ca_v\alpha_1$ subunits are responsible for the physiological and pharmacological properties of calcium channels, the trafficking and functioning of different types of VGCC require the auxiliary β and $\alpha_2\delta$ subunits (Ertel et al. 2000; Arikkath and Campbell 2003; Buraei and Yang 2010).

The β subunit ($Ca_v\beta$, 54 kDa) is an intracellular hydrophilic protein. There are four different types of $Ca_v\beta$ ($Cav\beta_{1-4}$), each with splice variants, encoded by four distinct genes. All four $Ca_v\beta$ share a common central core, whereas their N- and C-termini differ significantly. All four $Ca_v\beta$ dramatically enhance calcium channel currents when they are coexpressed along with the $Ca_v\alpha_1$ subunit in heterologous expression systems. $Ca_v\beta$ can also modulate the voltage-dependence, kinetics of activation and inactivation without affecting ion permeation (Obermair et al. 2008; Dolphin 2009; Karunasekara et al. 2009). $Ca_v\beta$ interacts with $Ca_v\alpha_1$ mainly through the β-interaction domain (BID) that binds with high-affinity to the α-interaction domain (AID) in the cytoplasmic loop of $Ca_v\alpha_1$ connecting the first two homologous repeats (De Waard et al. 1995; Witcher et al. 1995; Chen et al. 2004).

The γ subunit ($Ca_v\gamma$, 30 kDa) is an intracellular hydrophilic protein. There are eight different genes encoding $Ca_v\gamma$ subunits ($Ca_v\gamma_{1-8}$). Various $Ca_v\gamma$ subunits have been shown to affect kinetics and voltage-dependent gating of VGCC (Kang and Campbell 2003; Chen et al. 2007). $Ca_v\gamma_1$ was first cloned from muscle VGCC (Jay et al. 1990). Coexpression of $Ca_v\gamma$ subunit with L-type calcium channel subunits modulates Ca^{2+} peak current, activation and inactivation kinetics. This has been confirmed by subsequent studies in $Ca_v\gamma_1$ knockout mice (Arikkath et al. 2003), which show increased Ca^{2+} peak currents and altered inactivation kinetics compared with their age and sex matched wild type littermates (Freise et al. 2000).

In stagazer mutant mice, $Ca_v\gamma_2$ subunit levels are significantly reduced, and this change shifts calcium channel inactivation to more negative potentials. This deficit accounts for the distinctive phenotype, including head-tossing and ataxic gait (Letts et al. 1998).

All Ca_v1 and Ca_v2 channels contain transmembrane auxiliary $Ca_v\alpha_2\delta$ subunits (Felix 1999; Dolphin 2009). There are four subfamilies of $Ca_v\alpha_2\delta$ subunits ($Ca_v\alpha_2\delta_{1-4}$), each encoded by a unique gene, and the α_2 (143 kDa) and δ (24–27 kDa) peptides are cleaved then linked by disulfide bounds post-translationally (Felix 1999). When co-expressed along with $Ca_v\alpha_1$ and $Ca_v\beta$ subunits of Ca_v1 or Ca_v2 channels in heterologous expression systems, $Ca_v\alpha_2\delta$ subunits can dramatically increase calcium channel currents (Mori et al. 1991; Singer et al. 1991; Klugbauer et al. 1999, 2003; Gao et al. 2000; Davies et al. 2006). The enhancement is associated with the increased trafficking and retention of $Ca_v\alpha_1$ to the plasma membrane (Gurnett et al. 1997; Canti et al. 2005; Bernstein and Jones 2007). The systemic tissue distribution of $Ca_v\alpha_2\delta$ subunits has been analyzed at the mRNA and protein levels by different laboratories (Klugbauer et al. 1999; Hobom et al. 2000; Gong et al. 2001; Marais et al. 2001). $Ca_v\alpha_2\delta_1$ is abundantly expressed in excitable tissues such as the brain, heart, and muscles. $Ca_v\alpha_2\delta_2$ is expressed in various tissues with the highest levels in brain, heart, pancreas, and skeletal muscles. In a more restricted way, $Ca_v\alpha_2\delta_3$ expression levels are high in the brain, but low in the heart and skeletal muscles.

Since $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ are binding sites for gabapentin and pregabalin, which were originally designed as antiepilepsy drugs but have unexpected antineuropathic pain properties (Gee et al. 1996; Field et al. 2006), the contribution of $Ca_v\alpha_2\delta$ subunits, specially the $Ca_v\alpha_2\delta_1$ subunit, to pain processing has been studied extensively in the past decade.

15.3 Structure of $Ca_v\alpha_2\delta$ Subunits

Studies of transmembrane topology of $Ca_v\alpha_2\delta$ subunits have shown that the α_2 peptide is entirely extracellular (Brickley et al. 1995; Gurnett et al. 1996). The δ peptide is originally assumed to be transmembrane through a hydrophobic domain (Brickley et al. 1995; Gurnett et al. 1996). However, Davies et al. have recently reported that the δ peptide is attached to the membrane through a glycosylphosphatidylinositol linker (Davies et al. 2010). Even though $Ca_v\alpha_2\delta_2$ and $Ca_v\alpha_2\delta_3$ share only 56 and 30 % sequence homology with $Ca_v\alpha_2\delta_1$ respectively (Klugbauer et al. 1999), $Ca_v\alpha_2\delta$ subunits share important structure features including a similar transmembrane topology and heavy glycosylation at the extracellular domain (Klugbauer et al. 1999). Gurnett et al. have shown that both the disulfide bond and glycosylation in $Ca_v\alpha_2\delta_1$ play a critical role in enhancing $Ca_v2.1$ currents (Gurnett et al. 1996, 1997). Data from Western blot studies indicate that $Ca_v\alpha_2\delta_1$, $Ca_v\alpha_2\delta_2$, $Ca_v\alpha_2\delta_3$ and $Ca_v\alpha_2\delta_4$ have similar molecular weights: 200 kDa, 190 kDa, 166 kDa and 138 kDa, respectively (Marais et al. 2001). Splicing variants of $Ca_v\alpha_2\delta$ subunits

(five for $Ca_v\alpha_2\delta_1$, and three for $Ca_v\alpha_2\delta_2$), which differ by three to eight amino acid residues, greatly increase the proteome diversity of calcium channels. These splice variants are differentially expressed in cardiac tissue and brain (Klugbauer et al. 1999; Marais et al. 2001).

It has been shown that $Ca_v\alpha_2\delta$ binds to extracellular domains of $Ca_v\alpha_1$ subunit (Felix et al. 1997; Gurnett et al. 1997). One important domain in $Ca_v\alpha_2\delta$ subunits that has been identified through sequence homology is the highly conserved Von Willebrand factor type A domain (VWA, residues 253–430 of $Ca_v\alpha_2\delta_1$, and residues 294–472 of $Ca_v\alpha_2\delta_2$), which is also present in integrins. The VWA domain is extracellular, has binding sites for extracellular matrix proteins, and contains a metal ion-dependent adhesion site (MIDAS) motif (Whittaker and Hynes 2002). Only $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$, but not $Ca_v\alpha_2\delta_3$ or $Ca_v\alpha_2\delta_4$, subunits contain the MIDAS motif. Recent findings have suggested that $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ can both interact with $Ca_v\alpha_1$ subunit through the MIDAS motif and undergo an integrin-like switch, therefore, enhancing cell surface trafficking and currents of the calcium channel complex (Canti et al. 2005).

15.4 Pathophysiological Functions of $Ca_v\alpha_2\delta$ Subunit

15.4.1 Regulation of VGCC Expression

Numerous studies indicate that $Ca_v\alpha_2\delta$ subunits can markedly increase normal VGCC surface expression indicated by increased current amplitude in various in vitro heterologous expression systems, including Xenopus oocytes and mammalian cell lines (Mori et al. 1991; Singer et al. 1991; Shistik et al. 1995; Klugbauer et al. 1999, 2003; Gao et al. 2000; Hobom et al. 2000; Barclay et al. 2001; Canti and Dolphin 2003; Field et al. 2006; Davies et al. 2010). Mutation or overexpression of the $Ca_v\alpha_2\delta$ genes in vivo provides us with useful tools to characterize physiological and pathological roles of $Ca_v\alpha_2\delta_2$ in vivo. Spontaneous mutations in the $Ca_v\alpha_2\delta_2$ gene disrupt $Ca_v\alpha_2\delta_2$ expression in *ducky* mice (Brodbeck et al. 2002). Electrophysiological recording data have shown that the loss of $Ca_v\alpha_2\delta_2$ subunit in Purkinje cells of *ducky* mice results in a 35 % decrease in P-type calcium channel current amplitude, but has no effect on single P-type calcium channel conductance (Barclay et al. 2001). These results indicate that loss of $Ca_v\alpha_2\delta_2$ in vivo reduces VGCC surface expression. In contrast, $Ca_v\alpha_2\delta_1$ subunit overexpression in neuronal cells of transgenic mice results in ~60 % larger Ca^{2+} currents in dorsal root ganglion (DRG) sensory neurons, than that from their wild type littermates, which can be blocked by gabapentin in a concentration-dependent manner, supporting that increased $Ca_v\alpha_2\delta_1$ expression leads to elevated VGCC currents in sensory neurons (Li et al. 2006). Since $Ca_v\alpha_2\delta$ subunits do not change single-channel properties of VGCC such as conductance and open probability (Klugbauer et al. 2003), the increase in current amplitude is likely associated with a chaperoning effect of $Ca_v\alpha_2\delta$ subunits on membrane surface VGCC expression.

Once the calcium channel complex reaches the plasma membrane, $Ca_v\alpha_2\delta$ subunits also dramatically alter voltage-dependence and gating kinetics of VGCC. In general, $Ca_v\alpha_2\delta$ subunits shift voltage-dependent activation and inactivation of VGCC to more negative membrane potentials, and accelerate the inactivation kinetics of VGCC (Klugbauer et al. 2003). However, these effects may differ among individual $Ca_v\alpha_2\delta$ subunits (Hobom et al. 2000) and depending on $Ca_v\alpha_2\delta$ levels. In $Ca_v\alpha_2\delta_1$ overexpressing transgenic mice, increased $Ca_v\alpha_2\delta_1$ expression in sensory neurons leads to a shift of voltage-dependent activation to a more negative membrane potential compared with wild type neurons, an increase in voltage-dependence and rate of activation, and a decrease in voltage-dependent deactivation rate (Li et al. 2006). These findings support that elevated $Ca_v\alpha_2\delta_1$ levels also modulate VGCC kinetics.

How does $Ca_v\alpha_2\delta$ enhance calcium channel surface expression? One hypothesis is that a gabapentin binding site in $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ subunits has a chaperoning effect on VGCC as gabapentin intracellularly disrupts the process of $Ca_v\alpha_2\delta$ and Ca_{v2} trafficking, which could be prevented by a single mutation of the gabapentin binding site in $Ca_v\alpha_2\delta_1$ (R217A) and $Ca_v\alpha_2\delta_2$ (R282A) (Heblich et al. 2008). Alternatively, the VWA domain in the $Ca_v\alpha_2$ protein may interact with $Ca_v\alpha_1$ and thus enhance its trafficking to the plasma membrane. Mutations of three key amino acids (D300, S302, and S304) in the MIDAS motif of the VWA domain in $Ca_v\alpha_2\delta_2$ diminish $Ca_V1.2$, $Ca_V2.1$, $Ca_V2.2$ currents, probably through increased intracellular retention of the $Ca_v\alpha_1$ subunit (Canti et al. 2005).

15.4.2 Presynaptic Expression of $Ca_v\alpha_2\delta$ in Terminals of Sensory Neurons

Under normal conditions, $Ca_v\alpha_2\delta$ is expressed in sensory neurons in dorsal root ganglia, then undergoes anterograde transport to the presynaptic terminals in dorsal spinal cord. Dorsal rhyzotomy, which terminates the connection between dorsal root ganglia and dorsal spinal cord, results in about 50 % reduction in dorsal spinal cord $Ca_v\alpha_2\delta_1$ levels (Li et al. 2004). This indicates that, under normal conditions, $Ca_v\alpha_2\delta_1$ in dorsal spinal cord is expressed at both presynaptic and postsynaptic locations. A recent study provides the first direct evidence supporting that $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ increase P/Q VGCC accumulation at presynaptic boutons and enhance vesicle exocytosis and presynaptic function of VGCC (Hoppa et al. 2012).

15.4.3 $Ca_v\alpha_2\delta$ Functions Independent of Calcium Channel Activity

The functions of $Ca_v\alpha_2\delta$ have long been exclusively linked with VGCC. However, recent studies suggest that $Ca_v\alpha_2\delta$ may possess functions independent of their

association with VGCC. Data from a recent study have shown that $Ca_v\alpha_2\delta$ is the receptor for thrombospondin (TSP), an extracellular matrix protein secreted by astrocytes, in promoting central nervous system synaptogenesis (Eroglu et al. 2009). Neuronal $Ca_v\alpha_2\delta_1$ overexpression in transgenic mice results in increased excitatory synapse numbers in the brain. TSP treatment on retinal ganglion cells with $Ca_v\alpha_2\delta_1$ overexpression results in a 100 % increase in the number of synapses, which can be blocked by the $Ca_v\alpha_2\delta_1$ ligand gabapentin. L-, N- or P/Q-type VGCC blockers fail to inhibit TSP-induced synapse formation, suggesting that the roles of $Ca_v\alpha_2\delta$ in synapse formation are not likely associated with VGCC functions.

Consistent with this notion, Purkinje cells in *ducky* mice lacking $Ca_v\alpha_2\delta_2$ have abnormal synapse formation (Brodbeck et al. 2002). $Ca_v\alpha_2\delta_3$ null mutant drosophila embryos lack boutons in neuromuscular junctions of $Ca_v\alpha_2\delta_3$ mutant terminals due to missing ankyrin2-XL, a protein stabilizes synapses by anchoring cell surface proteins in synaptic terminals, that disturbs cytoskeleton arrangement (Kurshan et al. 2009). Boutons are restored by re-expressing $Ca_v\alpha_2\delta_3$ in $Ca_v\alpha_2\delta_3$ null embryos, suggesting that $Ca_v\alpha_2\delta_3$ is involved in the formation of nerve terminals. This process is unlikely to depend on VGCC-related actions since pore forming $Ca_v\alpha_1$ mutant embryos have normal ankyrin2 expression and boutons in nerve terminals (Brodbeck et al. 2002).

15.4.4 Implication of $Ca_v\alpha_2\delta$ Dysregulation in Pain Processing

Three types of $Ca_v\alpha_2\delta$ ($Ca_v\alpha_2\delta_1$, $Ca_v\alpha_2\delta_2$ and $Ca_v\alpha_2\delta_3$) mRNA are identified in primary sensory neurons in DRG (Cole et al. 2005). $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ mRNAs are highly expressed in small DRG neurons but with low expression in large DRG neurons, whereas $Ca_v\alpha_2\delta_3$ mRNA is only present in large DRG neurons (Yusaf et al. 2001). These data suggest that $Ca_v\alpha_2\delta$ subunits may play unique roles in sensory information processing.

The involvement of $Ca_v\alpha_2\delta$ in pain processing is further supported by pharmacology data indicating that gabapentinoids, including gabapentin and pregabalin, have high binding affinity for VGCC $Ca_v\alpha_2\delta_1$ and $Ca_v\alpha_2\delta_2$ subunits (Gee et al. 1996; Marais et al. 2001), and anti-nociception properties in animal models (Hwang and Yaksh 1997; Luo et al. 2001, 2002) and patients (Dworkin and Kirkpatrick 2005; Guay 2005; Zareba 2005). Mutations at the gabepentin binding site within the α_2 peptide (R217A) eliminate gabapentin binding and its anti-nociceptive actions (Field et al. 2006), further confirmed that binding of gabapentinoids to $Ca_v\alpha_2\delta$ proteins may underlie the anti-nociceptive actions of these drugs.

Under pathological conditions that lead to the development of behavioral hypersensitivities, such as peripheral nerve injury and diabetic neuropathies, $Ca_v\alpha_2\delta_1$ upregulation has been reported in dorsal root ganglia and dorsal spinal cord of pain models that correlates with the development of thermal and mechanical hypersensitivities (Luo 2000, 2004; Luo et al. 2001, 2002; Newton et al. 2001; Yusaf et al. 2001; Li et al. 2006). Interestingly, $Ca_v\alpha_2\delta_2$ and $Ca_v\alpha_2\delta_3$ mRNA are

Table 15.1 Dysregulation of voltage gated calcium channel $Ca_v\alpha_2\delta$ subunit in pain models

$Ca_v\alpha_2\delta$ subunit	Dysregulation	Model	Behavioral hypersensitivity	References
$Ca_v\alpha_2\delta_1$	↑ in DRG, DSC	SNL SNTx CCI DNP Paclitaxel-evoked neuropathy SCI Partial sciatic nerve injury	Tactile allodynia, mechanical and thermal hyperalgesia	Luo et al. (2001, 2002), Newton et al. (2001), Yusaf et al. (2001), Valder et al. (2003), Li et al. (2004), Xiao et al. (2007), Bauer et al. (2009), Kim et al. (2009), and Boroujerdi et al. (2011)
$Ca_v\alpha_2\delta_2$	↓ in DRG (mRNA)	SNL	Tactile allodynia	Bauer et al. (2009)
$Ca_v\alpha_2\delta_3$	↓ in DRG (mRNA)	SNL	Tactile allodynia	Bauer et al. (2009)
$Ca_v\alpha_2\delta_4$	ND			

SNL spinal nerve ligation, *SNTx* spinal nerve transection, *CCI* chronic constriction injury of the sciatic nerve, *DNP* Diabetic neuropathy, *SCI* spinal cord injury. *ND* not determined

downregulated after peripheral nerve injury, suggesting a dominant role of $Ca_v\alpha_2\delta_1$ over $Ca_v\alpha_2\delta_2$ and $Ca_v\alpha_2\delta_3$ in peripheral nerve injury-induced pain processing (Bauer et al. 2009) (Table 15.1).

This is confirmed by in vivo findings that $Ca_v\alpha_2\delta_1$ upregulation is required for the onset (Boroujerdi et al. 2008) as well as maintenance of neuropathic pain states (Luo et al. 2001); The antihyperalgesic effects of gabapentin are correlated with upregulation of $Ca_v\alpha_2\delta_1$ subunit in neuropathic pain models (Luo et al. 2002); Blocking injury signals that trigger $Ca_v\alpha_2\delta_1$ upregulation or blocking injury-induced $Ca_v\alpha_2\delta_1$ upregulation directly in a nerve injury model prevent the development of neuropathic pain states (Boroujerdi et al. 2008).

15.4.5 Presynaptic Modulation of Sensory Pathways by Abnormal $Ca_v\alpha_2\delta_1$ Expression

How does peripheral nerve injury-induced upregulation of $Ca_v\alpha_2\delta_1$ proteins contribute to neuropathic pain states? It has been shown that injury-induced upregulation of $Ca_v\alpha_2\delta_1$, but not $Ca_v\alpha_2\delta_2$, proteins in DRG are translocated to presynaptic terminals of sensory afferents in dorsal spinal cord (Li et al. 2004; Bauer et al. 2009). Several lines of evidence support that upregulated $Ca_v\alpha_2\delta_1$ at the presynaptic terminals of sensory afferents in dorsal spinal cord plays a critical role in mediating dorsal horn neuron sensitization and pain processing. (1) Only protein, but not mRNA, levels are upregulated in spinal cord suggesting that injury-induced

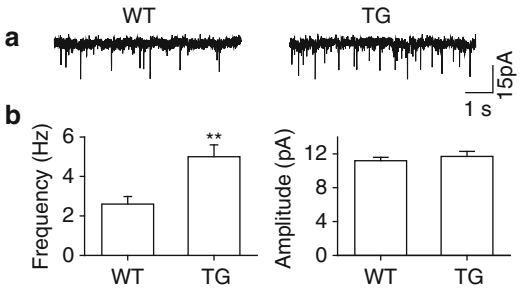

Fig. 15.1 Increased frequency, but not amplitude, of AMPA receptor mediated mEPSCs in dorsal spinal cord neurons of the $Ca_v\alpha_2\delta_1$ transgenic mice. (**a**) Representative traces of mEPSCs from dorsal spinal cord of wild type (WT) and $Ca_v\alpha_2\delta_1$ transgenic (TG) mice, respectively. (**b**) Summary of mEPSC frequency (*left*) and amplitude (*right*) from WT and TG mice, respectively. Data presented are means ± SEM from at least 15 neurons in each group. ** $p < 0.01$ compared with WT neurons by Students' *t* test

$Ca_v\alpha_2\delta_1$ dysregulation mainly occurs at the DRG level, which results in enhanced anterograde axonal transport of the elevated $Ca_v\alpha_2\delta_1$ to the presynaptic terminals of sensory afferents in dorsal spinal cord (Luo et al. 2001; Bauer et al. 2009). (2) Dorsal rhyzotomy that interrupts the anterograde axonal transport of $Ca_v\alpha_2\delta_1$ can block injury-induced $Ca_v\alpha_2\delta_1$ upregulation in dorsal spinal cord and reverse neuropathic pain states (Li et al. 2004). (3) Intrathecal $Ca_v\alpha_2\delta_1$ antisense oligodeoxynucleotide treatment abolishes injury-induced $Ca_v\alpha_2\delta_1$ upregulation in dorsal spinal cord, not in DRG, which correlates with a reversal of neuropathic pain states (Li et al. 2004). (4) Intrathecal injections with glutamate receptor antagonists eliminate behavioral hypersensitivity in spinal nerve ligated rats with $Ca_v\alpha_2\delta_1$ upregulation in DRG and dorsal spinal cord, and $Ca_v\alpha_2\delta_1$-overexpressing mice (Chaplan et al. 1997; Nguyen et al. 2009), suggesting that $Ca_v\alpha_2\delta_1$ mediates behavioral hypersensitivity by facilitating glutamate release at the spinal level. (5) Biochemical data indicate that $Ca_v\alpha_2\delta_1$ can regulate the evoked release of neurotransmitters, such as glutamate, GABA, Substance P, by enhancing the function of presynaptic VGCC, which is sensitive to blockade by gabapentinoids (Quintero et al. 2011). (6) Electrophysiological data indicate that the frequency, but not amplitude, of glutamate (AMPA) receptor-mediated miniature excitatory postsynaptic currents (mEPSC) is increased in $Ca_v\alpha_2\delta_1$-overexpressing transgenic mice (Nguyen et al. 2009) (Fig. 15.1). Since increased frequency of AMPA-receptor mediated mEPSC is a reflection of increased presynaptic release of glutamate, this suggests that elevated $Ca_v\alpha_2\delta_1$ promotes presynaptic glutamate release at the spinal cord level that, in turn, causes dorsal horn neuron sensitization, and behavioral hypersensitivity.

Using immunostaining techniques, Bauer el al. have reported that spinal nerve ligation injury leads to increased $Ca_v\alpha_2\delta_1$ immunoreactivity in axons of the fasciculus gracilis ascending from injured DRG rostrally up to the brainstem (Bauer et al. 2009). Chronic pregabalin treatment in the spinal nerve injured animals reduces this axonal increase of $Ca_v\alpha_2\delta_1$ immunoreactivity when compared with saline control

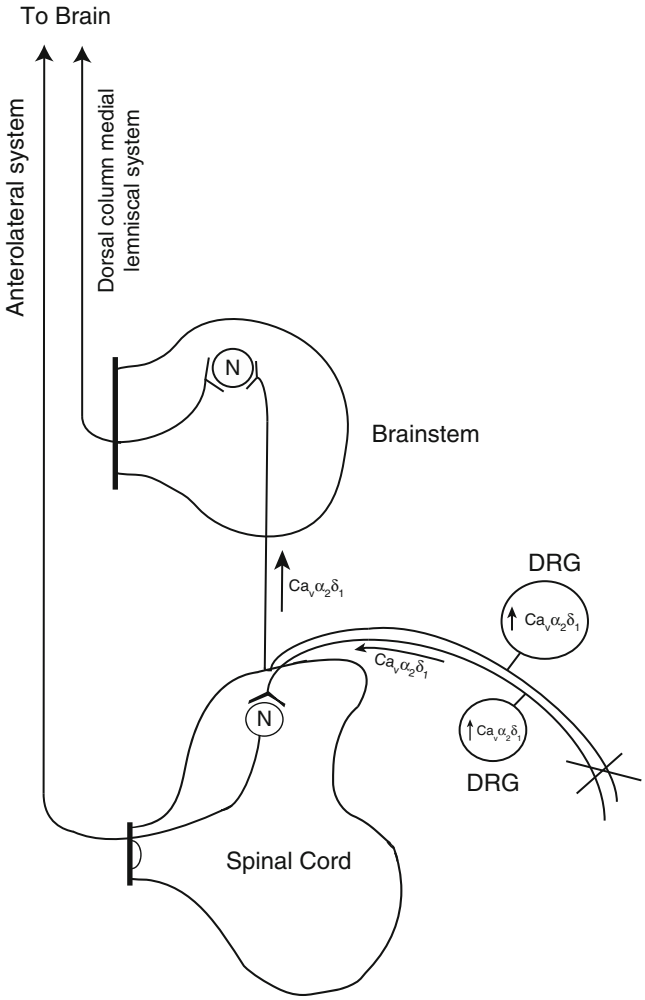

Fig. 15.2 Possible influence of elevated $Ca_v\alpha_2\delta_1$ at different locations along the sensory pathway. Schematic illustration showing how injury induced upregulation of $Ca_v\alpha_2\delta_1$ in DRG could be translocated to multiple locations along the sensory pathway, thus affect presynaptic neurotransmission at these sites. *N* neuron, *X* nerve injury

treatment, suggesting that injury-induced DRG $Ca_v\alpha_2\delta_1$ expression could reach presynaptic terminals of sensory afferents at the lower brainstem level to regulate local presynaptic neurotransmission. This change could affect the excitability of postsynaptic projection neurons sending ascending axons rostrally along the dorsal column medial lemniscal system (Fig. 15.2). In vivo or in vitro electrophysiological recording at that level from peripheral nerve injured animals is warranted to further

test this hypothesis. In vitro studies have suggested that once in the presynaptic terminals, $Ca_v\alpha_2\delta_1$ proteins modulate presynaptic neurotransmission through two possible molecular mechanisms. First, elevated $Ca_v\alpha_2\delta_1$ proteins could increase the membrane expression of presynaptic VGCC. Second, elevated $Ca_v\alpha_2\delta_1$ proteins could increase release probability of neurotransmitter by presumably configuring presynaptic VGCC more favorable for driving exocytosis. The latter requires the presence of the MIDAS motif within the predicted VWA domain of $Ca_v\alpha_2\delta_1$ proteins (Hoppa et al. 2012). Whether similar mechanisms occur in vivo remains to be explored.

Alternatively, $Ca_v\alpha_2\delta_1$ proteins may modulate sensory information processing through activities unrelated to VGCC. Recently, it has been shown that $Ca_v\alpha_2\delta_1$ proteins are critical in promoting excitatory synaptogenesis by serving as neuronal receptors for TSP (Eroglu et al. 2009; Kurshan et al. 2009). VWA domain within $Ca_v\alpha_2\delta_1$ is critical for its interaction with TSP proteins. Importantly, TSP4 is recently identified as a pro-nociceptive factor, which is overly expressed in activated astrocytes in dorsal spinal cord post peripheral nerve injury that leads to enhancing pre-synaptic neurotransmission, dorsal horn neuron sensitization and neuropathic pain processing (Kim et al. 2012). Together, it is likely that increased $Ca_v\alpha_2\delta_1$ in dorsal spinal cord presynaptic terminals of sensory afferents interacts with TSP4 secreted from activated astrocytes to promote formation of excitatory synapses, which can lead to exaggerated neurotransmitter release upon peripheral stimulation and pain sensations. Further studies are required to reveal this potential mechanism of pain processing.

15.4.6 Descending Modulatory Pathways Regulated by $Ca_v\alpha_2\delta_1$

Descending pain modulatory pathways from the cortex, thalamus and brainstem send both inhibitory and facilitatory inputs to the dorsal horn to modulate sensory input from primary afferents in dorsal spinal cord. The release of serotonin, norepinephrine and endogenous opioids from descending pathways can modulate the release of excitatory neurotransmitters, excitatory and inhibitory interneuron activity as well as projection neuron sensitivity at the spinal level. Impairment of these descending modulation pathways often leads to development of chronic pain states.

$Ca_v\alpha_2\delta$ subunits are also expressed in discrete supraspinal regions along descending modulatory pathways (Cole et al. 2005). It has been shown that intracerebroventricular (i.c.v.) administration of gabapentin and pregabalin can reduce thermal and mechanical hypersensitivities in a pain model of peripheral nerve injury without affecting acute thermal and mechanical nociception. These anti-hyperalgesic effects of gabapentinoids correlate with the accelerated spinal turnover of noradrenaline. Following noradrenaline depletion by intracisternal

injection of 6-hydroxydopamine, i.c.v. administration of pregabalin has no effect on thermal and mechanical hypersensitivities. These findings support that gabapentinoids activate the descending noradrenergic pain inhibitory pathway supraspinally in alleviating pain states post nerve injury (Tanabe et al. 2005; Takeuchi et al. 2007a, b).

Similarly, Hayashida et al. have injected gabapentin directly into locus coeruleus (LC) in the pons, and reported that gabapentin reduces behavioral hypersensitivity in spinal nerve ligated rats in a dose-dependent manner, which can be blocked by intra-LC injection of idazoxan, an α2-adrenoceptor antagonist (Hayashida et al. 2008). In addition, data from an in vitro patch clamp recording in LC slices have shown that bath application of gabapentin dose-dependently inhibits $GABA_A$ receptor-mediated, evoked inhibitory postsynaptic currents (IPSC) with increased paired-pulse ratio from peripheral nerve injury mice, but has no effect on IPSC from sham control mice. In contrast, gabapentin treatments do not affect glutamate-mediated evoked excitatory postsynaptic currents (EPSC) in LC of nerve injury mice (Takasu et al. 2008). The authors concluded that gabapentin inhibits GABAergic synaptic transmission in LC through a presynaptic mechanism and subsequently removes inhibitory effects on LC neurons and activates descending noradrenergic inhibition under a neuropathic pain inducing condition (nerve injury). Together, these findings suggest that gabapentin acts directly or indirectly on noradrenergic neurons in the brainstem to stimulate descending inhibition after peripheral nerve injury. This is supported by a clinical study in human indicating that oral gabapentin before surgery significantly increases norepinephrine concentration in cerebrospinal fluid (Hayashida et al. 2007). Because $Ca_v\alpha_2\delta_1$ subunit is the only known receptor for gabapentin and pregabalin, and is dysregulated after peripheral nerve injury, it is possible that gabapentin and pregabalin modulate a noradrenergic descending pathway through binding to the $Ca_v\alpha_2\delta_1$ subunit at the supraspinal level.

Recent studies also suggest that activation of descending 5-HT_3 facilitatory pathway is required for the processing of nociceptive signals in normal and nerve injured animals, as well as the state-dependent inhibitory actions of pregabalin in late stages of nerve injury in a neuropathic pain model (Bee and Dickenson 2008). Ablation of descending facilitatory cells expressing the mu-opioid receptor in rostral ventromedial medulla renders pregabalin ineffective in inhibiting spinal neuron activity, which can be restored by intrathecal injection of a $5HT_3$ receptor agonist to mimic the descending drive at the spinal level (Bee and Dickenson 2008). This suggests that injury-induced $Ca_v\alpha_2\delta_1$ dysregulation, which usually occur in a late stage of nerve injury (Li et al. 2004), may mediate neuropathic pain states through a 5-HT_3 receptor-dependent pathway. To test this hypothesis, we have examined if the descending 5-HT_3 facilitatory pathway is involved in mediating pain states induced by $Ca_v\alpha_2\delta_1$ upregulation at the spinal level by comparing the effects of a 5-HT_3 receptor antagonist in behavioral hypersensitivities in the neuropathic pain model of spinal nerve ligation and $Ca_v\alpha_2\delta_1$ overexpressing transgenic mice. Our findings have indicated that intrathecally, but not systematically, injected ondansetron, a 5-HT_3 receptor antagonist, can block dose-dependently mechanical and thermal

hypersensitivities in both the nerve injury model and injury-free $Ca_v\alpha_2\delta_1$ overexpressing transgenic mice (Chang et al. 2012). Together, these findings support that the serotonergic descending facilitation pathway is involved in central sensitization and pain states mediated by $Ca_v\alpha_2\delta_1$ upregulation, either induced by peripheral nerve injury or transgenic $Ca_v\alpha_2\delta_1$ overexpression, at the spinal level.

15.5 Perspectives

Structure, cellular/molecular biology, and pathophysiological functions of $Ca_v\alpha_2\delta$ subunits have been extensively studied in the last two decades. Moreover, a large body of emerging evidence indicates that $Ca_v\alpha_2\delta$ subunit is a multifunctional protein. It regulates not only pathophysiological functions of VGCC, but also VGCC-independent functions. The following important questions regarding the functions of $Ca_v\alpha_2\delta$ subunits in disease states remain to be elucidated.

1. What is the functional implication of $Ca_v\alpha_2\delta$ dysregulation in modulation of VGCC trafficking and functions, facilitation of synaptic neurotransmission, and alterations in neural circuits in disease states?
2. In addition to TSP and ankyrin2-XL, which other proteins interact with $Ca_v\alpha_2\delta$ under different pathological conditions? What are the signaling pathways underlying $Ca_v\alpha_2\delta$ mediated pathological conditions such as pain processing?
3. Is $Ca_v\alpha_2\delta$ dysregulation in sensory neurons cell-type specific? If so, what is the implication of cell-type specific $Ca_v\alpha_2\delta$ dysregulation and its neuraxial distribution in mediating modality specific behavioural hypersensitivity?
4. What are the factors and signalling pathways involved in mediating $Ca_v\alpha_2\delta$ dysregulation under pathological conditions?

Discoveries leading to the understanding of these questions will provide us with a new insight into disorders related to $Ca_v\alpha_2\delta$ dysregulation and lead to the development of new and target specific medications for management of disorders involving $Ca_v\alpha_2\delta$ dysregulation.

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Index

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